Master of Sciences in Bioengineering

Molecularly Imprinted Polymer grafted onto Mesoporous Carbon Nanoparticles for selective removal of 5-fluorouracil from water samples

Thesis

of

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To Cosmos…

"Reality is merely an illusion, albeit a very persistent one."

Albert Einstein

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Abstract

Cytostatic drugs are pharmaceutically active compounds (PhACs) used to treat cancer that recently have been found in wastewaters (WWs) and even in surface waters (SFs). Accordingly to ecotoxicity data, 5-fluorouracil (5-FU) is the cytostatic that has been found to be of most concern. The drug isn't fully metabolized and about 15% of the administered dose is excreted in urine as intact drug. In the present work, it was produced a molecularly imprinted polymer (MIP) intended to be selective to 5-FU that can be applied in water samples. MIP was grafted onto mesoporous carbon nanoparticles (MCN), which were produced by hydrothermal reaction and hard templating with mesoporous silica nanoparticles (MSNs). Results about particles textural properties revealed that the procedure requires some optimizations to perform. Binding experiments were performed thanks to a HPLC analytical method development and validation. A C_{18} column and a mobile phase of water/acetonitrile (97/3, v/v) at a flow rate of 1.0 mL/min were used to determine 5-FU in water samples. The linearity was 1.000, in a range of 0.03 – 25 mg/L. The limits of quantification and detection were 0.03 and 0.01 respectively. The relative standard deviation (RSD) was below 7.0% for the evaluation of precision and the recovery was in tha range of 95 – 107%. Dynamic (kinetics) and static (isotherms) sorption studies were performed and the 5FU-MCN-MIP presented an adsorption capacity of 37 mg/g but further studies should be done. This was an introduction to a new technology of selective removal of cytostatics from water samples and there are still improvements to perform. This methodology is promising for future application in real situations of wastewaters treatment plants (WWTPs).

Graphical Abstract

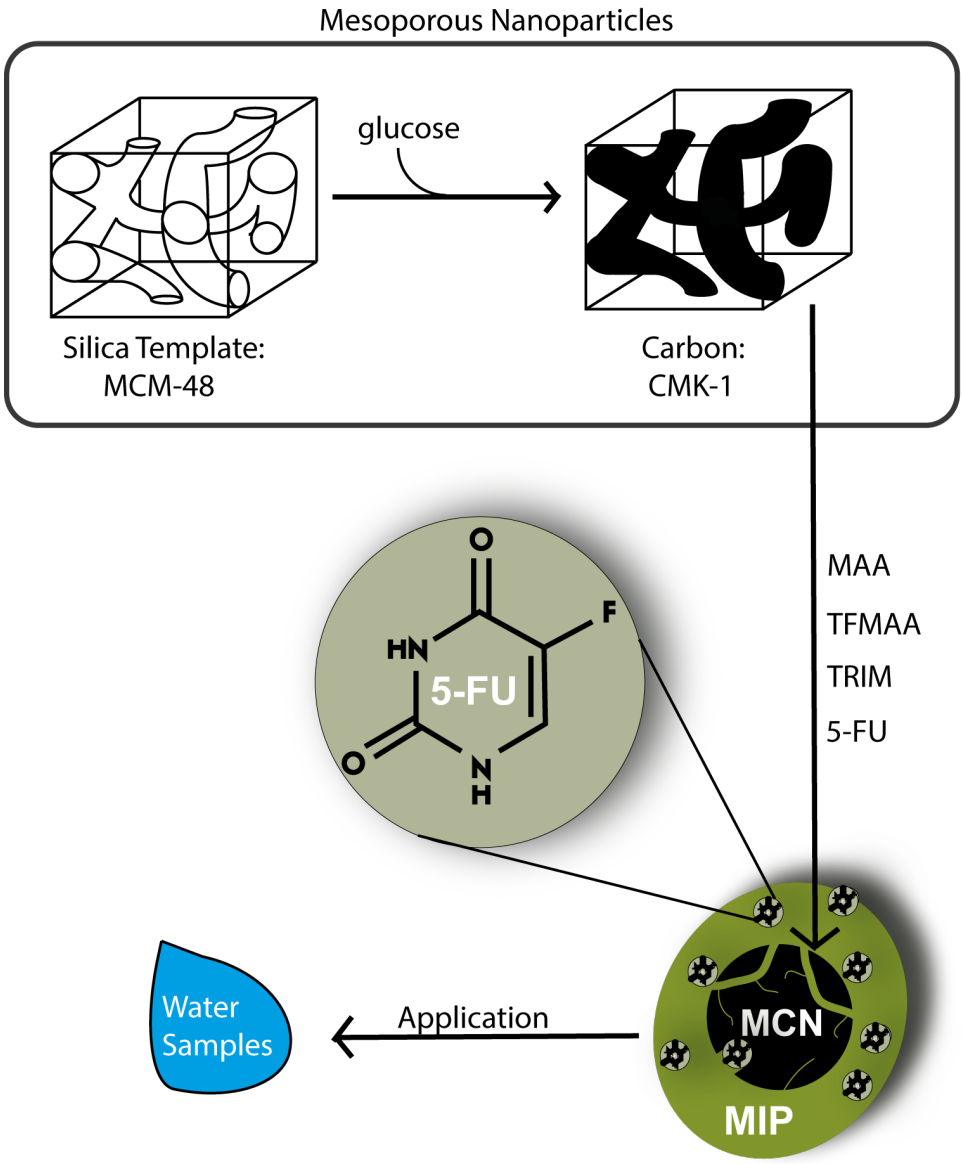


Table of Contents

Acknowledgements v

Abstract vii

Graphical Abstract viii

Table of Contents ix

List of figures xi

List of tables xii

Abbreviations xiii

1 Introduction 1

1.1 Main Objectives 4

1.2 Thesis Organization 4

2 State of Art 5

2.1 Cytostatics: 5-fluorouracil 5

2.1.1 Analytical methods 8

2.2 Molecularly Imprinted Polymers (MIPs) 10

2.2.1 Methods for MIP synthesis 11

2.3 MIPs and 5-FU 13

3 Technical Description and Discussion of Results 15

3.1 Materials 15

3.2 Equipment 15

3.3 Experimental 16

3.3.1 MSNs synthesis 16

3.3.2 MCNs synthesis 17

3.3.3 MCNs functionalization 18

3.3.4 MIP synthesis 19

3.3.5 Analytical method 19

3.3.6 Binding Experiments 20

3.4 Results and discussion 21

3.4.1 Textural properties 21

3.4.2 Evaluation of Functionalization 23

3.4.3 Analytical method 26

3.4.4 Sorption kinetics 27

3.4.5 Sorption isotherms 28

3.4.6 Adsorption conditions 30

4 Conclusions 31

4.1 Future work 32

References 33

Appendix 1 - Metabolism pathway of 5-FU 39

Appendix 2 - Review of Analytical Methods 41

Appendix 3 - Table Protocol 44

List of figures

Figure 1. Scheme showing possible sources and pathways for the occurrence of PhACs residues in the aquatic environment. 1

Figure 2. The metabolism pathways of 5-FU in humans. Adapted from [24]. 5-fluoro-5,6-dihydrouracil (5-FUH_2), 5-fluorouridine (5-FUrd), 5-fluoro-2'-deoxyuridine (5-FdUrd), 5-fluorouridine-5'-monophosphate (FMUP). 3

Figure 3. Bioactivation pathway of capecitabine. Enzymes: carboxylesterase (CES); cytidine deaminase (CDA); thymidine phosphorylase (dThdPhase); dihydropyrimidine dehydrogenase (DPD); dihydropyrimidinase (DHP); B-alanine synthase (BUP). 6

Figure 4. Illustration of interaction between 5-FU and MAA. 14

Figure 5. CTAB micelle designed on MarvinSketch Software v.6.2 (A) and two possible interactions between silica source and micelle adapted from [59] (B). 16

Figure 6. Mechanism of formation of carbon particles by hydrothermal carbonization. 17

Figure 7. The routes of introducing vinyl groups and grafting MIP onto the surface of the MCNs. 18

Figure 8. Nitrogen adsorption-desorption isotherm of MSNs. 21

Figure 9. Enlarged SEM image of the mixture of MSNs and carbon microspheres via hydrothermal synthesis using blank MCM-48 as template (the small nanoparticles with size of 150nm are MSNs and the sizes of the totally separated carbon microspheres are about 2 μm). 23

Figure 10. FTIR spectra of MSNs and its amino-funcionalzation and MCNs and its carboxyl functionalization. 24

Figure 11. Colour difference of 5FU-MCN-MIP (left) and MCN-NIP (right) in aqueous solution. 25

Figure 13. The adsorption kinetic curve for 5FU-MCN-MIP at 25 mg/L of 5-FU. 28

Figure 14. The adsorption kinetic curve for MCN-NIP at 25 mg/L of 5-FU. 28

Figure 15. Binding results for the 5FU-MCN-MIPs and MCN-NIP in aqueous solution. 29

Figure 16. Adsorption comparison testing different adsorption conditions. 30

Figure 17. 5FU-MCN-MIP applied in wastewater treatment plant (WWTP). 32

List of tables

Table 1. Ocurrence of 5-FU in wastewaters. 7

Table 2. Consumption and predicted environmental concentration of 5-FU and CAP. 8

Table 3. Methods for MIP preparation [9, 31, 33]. 11

Table 4. Studies reporting 5-fluorouracil-templated MIPs (5FU-MIPs). 13

Table 5. Textural properties of particles. 22

Table 6. Method development and validation parameters. 27

Abbreviations

5-FU	5-fluoruracil
5-BU	5-bromouracil
ACN	Acetonitrile
APTES	3-aminopropyltriethoxysilane
Aq. NH_3	Ammonium hydroxide
ATC	Anatomical therapeutic chemical
BAP	2,6-bis(acrylamido)pyridine
BUP	β-alanine synthase
CAP	Capecitabine
CAS	Chemical abstracts services
CDA	Cytidine deaminase
CE	Capillary electrophoresis
CES	Carboxylesterase
CRC	Colon rectal cancer
CTAB	Hexadecyltrimethylammonium bromide
DAD	Diode array detector
DHP	Dihydropyrimidinase
DMAP	4-dimethylaminopyridine
DPD	Dihydropyrimidine dehydrogenase
dThdPhase	Thymidine phosphorylase
EGDMA	Ethylene glycol dimethacrylate
Et_3N	Triethylamine
EtOH	Ethanol
FBAL	α-fluoro-β-alanine
FQ	Fluorquinolone
FTIR	Fourier-Transform infrared spectroscopy
GC	Gas chromatography
HF	Hydrofluoric acid
HPLC	High pressure liquid chromatography
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methacrylic acid
MBAA	N,N-methylenebisacrylamide
MCN	Mesoporous carbon nanoparticle

MIP	Molecularly imprinted polymer
MIT	Molecular imprinting technology
MS	Mass-spectrometry
MSN	Mesoporous silica nanoparticle
OFL	Ofloxacin
PEC	Predicted environmental concentration
PhACs	Pharmaceutically active compounds
RP	Reversed phase
RSD	Relative standard deviation
SEM	Scanning electron microscopy
$SOCl_2$	Thionyl chloride
SPE	Solid-phase extraction
SW	Surface water
TEM	Transmission electron microscopy
TEOS	Tetraethylorthosilicate
TFMAA	Trifluoromethylacrylic acid
THF	Tetrahydrofuran
TRIM	Trimethylolpropane trimethacrylate
WW	Wastewater
WWTP	Wastewater treatment plant
XRD	X-ray powder diffraction
β-CD	β-ciclodextrin

1 Introduction

The production of pharmaceuticals for human and veterinary treatments has increased during the past decades. The contamination of the environment by pharmaceutically active compounds (PhACs) has been recognized as an emerging issue since the 1970s, when they were detected for the first time in aquatic environment [1-6]. However, it was just in the late 1990s that an exponential growth in the number of publications about the presence, concentration, fate, degradation and ecotoxicological effect of PhACs in the environment was verified [5, 7].

Heberer, in 2002, published a review [4] that highlights the occurrence of more than 80 PhACs in the aquatic environment practically all around the globe including surface waters and drinking water systems. *Figure 1* shows possible sources and pathways for the occurrence of PhACs in the aquatic environment. The major problem isn't the disposal of unused medication via the toilet but the PhACs not completely metabolized in the organism, which are renal or fecal excreted by patients [4, 8, 9]. Despite of PhACs have been detected at levels below their acute toxicity [2, 4-6, 8, 10, 11], the chronic exposure to such compounds might affect humans and cause adverse effects in key organisms of ecosystems. Subsequently, it is important to target and quantify pharmaceuticals with environmental significance and assess their ecotoxicological risk [2, 5, 6, 8-13].

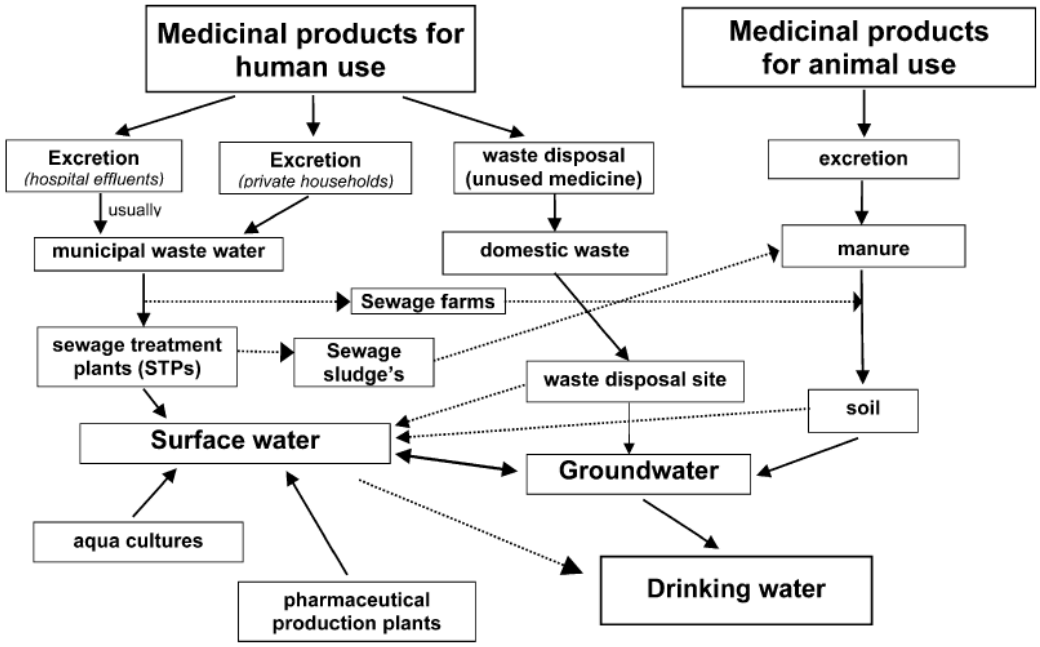


Figure 1. Scheme showing possible sources and pathways for the occurrence of PhACs residues in the aquatic environment. Adapted from [4].

Oldenkamp et al. tried to predict the risk of human health due to exposure to two different groups of PhACs: antibiotics and antineoplastic (or anticancer) drugs [14]. The authors presented different graphical representations about the risks inherent to exposure to these two groups of PhACs in Europe. The health risk obtained due to exposure to antibiotics is higher than for anticancer drugs but this comparison can't be truly made because the environmental picture for the second compounds is far from being completed. Antibiotics and even hormones have been studied intensively but antineoplastic drugs have received little attention, maybe due to their low environmental levels, which only recently, thanks to developments in analytical instrumentation, have been possible to address (from 10 ng/L in 1998 to 1 ng/L in 2011) [1, 4, 7, 11, 15].

Over the past decade the use of cytostatics - a therapeutic class of antineoplastic drugs - has increased considerably [16-18]. The action mechanism of these anticancer drugs is inhibition of cell growth leading cells to death [1, 7, 11, 18-20]. Antibiotics are also used in large quantities but cytostatics are potentially more toxic compounds because of their carcinogenic, mutagenic or embryotoxic properties which represent a potential risk for humans and the environment, especially for aquatic non-target species [1, 2, 4, 5, 9, 18, 21, 22]. Strong et al. proved this high cytotoxic potential when observed biochemical alterations in MCF-7 cells at very low concentrations (10^{-12} M – representative of concentrations observed in surface waters) of a cytostatic compound called cyclophosphamide [11]. More information about this group of PhACs is given in the section of state of art about cytostatics with special emphasis on 5-fluorouracil (5-FU) - the cytostatic selected for this study. 5-FU was chosen because it is a well known and one of the most frequently used cytostatic compound and may, therefore, be regarded as one of the pilot substances for environmental contamination [21]. Also, accordingly to ecotoxicity data, 5-FU has been found to be of most concern, with EC_{50} vales <1 mg/L (classified as "very toxic to aquatic organisms" by EU-Directive 93/67/ECC - EC 1996) [11]. The drug isn't fully degraded in the liver and the metabolites are excreted as carbon dioxide via respiration and as urea, α-fluoro-β-alanine (FBAL), α -fluoro-β-guanidopropionic acid and α-fluoro-β-ureidopropionic acid in urine. About 15% of the administered dose is excreted in urine as intact drug within 6 h. Approximately 80% is catabolized and excreted in urine mainly as FBAL [23, 24]. *Figure 2* shows the metabolism pathway of 5-FU in humans but a detailed scheme is in *Appendix 1*. In the section already mentioned of state of art, detailed information is presented about the occurrence of 5-FU in wastewater treatment plants (WWTPs).

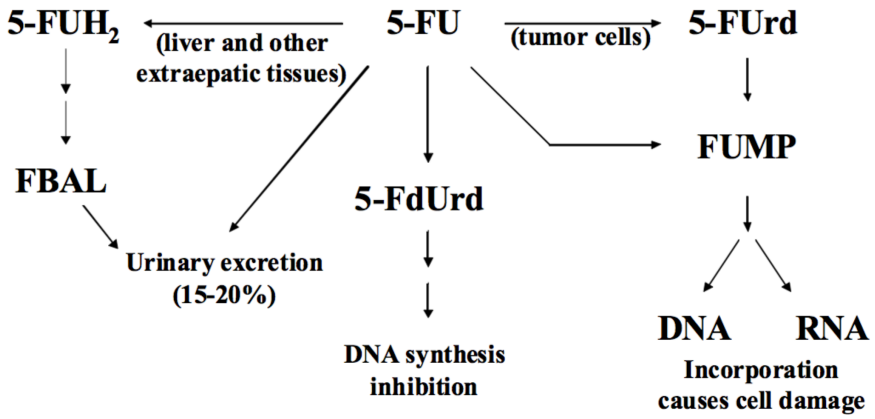


Figure 2. The metabolism pathways of 5-FU in humans. Adapted from [24]. 5-fluoro-5,6-dihydrouracil (5-FUH$_2$), 5-fluorouridine (5-FUrd), 5-fluoro-2'-deoxyuridine (5-FdUrd), 5-fluorouridine-5'-monophosphate (FMUP).

Standardized unit processes are available for different WWTPs to remove PhACs from polluted water, including chemical degradation, biological oxidation, physical adsorption, sedimentation and filtration. As anticancer drugs coexist with more abundant pollutants, the water treatment can be inefficient to remove all pollutants at the same time. For example, 5-FU has been detected in Hospital and Municipal wastewaters of Europe in the range of 0.005 – 124 µg/L [3, 16, 21]. Assuming the best removal rates in WWTPs, 5-FU predicted concentration in fluvial waters is higher than 0.9 ng/L [2, 11]. A preferable and economical way may be the selective removal of cytostatics [8].

Some carbon materials have been reported due their high adsorption capacity, but for higher selectivity - important for a matrix with many different pollutants - there is a practical interest in molecularly imprinted polymers (MIPs) [8-10, 25]. MIPs are synthesized by molecular imprinting technology (MIT). MIT allows formation of selective sites in a polymer matrix with memory of the shape, size and functional groups of the template molecules. So, MIPs are synthetic receptors with high-affinity sites that can selectively recognize a target compound, based on its shape, size, or functional group distribution. These receptors are promising due to their easy preparation, thermal stability, chemical inertness and long shelf life at room temperature and humidity [8-10, 26-34].

There is a technique being developed by Xie and co-workers [35], which combines MIPs with activated sludge. They reported enhanced degradation of trace highly toxic organic pollutants by using MIPs and activated sludge together. Also, MIPs were more suitable for enhanced biodegradation than non-selective sorbents as activated carbon. This is a simple,

effective, environmentally friendly application of MIPs for removal of PhACs from contaminated water that can be hereafter applied in wastewater treatment plants [10, 35].

In respect to MIPs selective to 5-FU, it was found in literature 4 different works but any of them for environmental applications. These works are presented in the section of state of art about MIPs.

1.1 Main Objectives

The real and accurate environment and human health risk of cytostatic drugs isn't clear and it is better to be sure that some cautions are being taken. The aim of this work was to produce a synthetic nano MIP highly selective for 5-FU (5FU-MIP) to be applied in water samples remediation. Also, it was developed an analytical method to further test the sorption capacity (k') of 5FU-MIP. 5-FU represents a good starting model for future application of the methodology here elaborated for other cytostatic drugs. The nano support material where MIP is grafted, besides being different from other 5FU-MIPs, is pointed out for great biocompatibility and good hydrodynamic behavior [8]. For the best of author knowledge this is the first study producing a 5FU-MIP for water samples remediation. Also, the methodology of nano MIP synthesis is an innovational technique in MIP production.

1.2 Thesis Organization

The background was introduced and the main objectives were established. In the next section – state of art - cytostatics are introduced with special attention on 5-FU characteristics and analysis methods. Also, different MIP method productions are shortly discussed because, after reviewing what was already made relating MIPs and 5-FU, it is presented a method for selective removal of 5-FU from water samples. The method leads to mesoporous silica nanoparticles (MSNs) and mesoporous carbon nanoparticles (MCNs), which will be also referenced in the following section.

Materials and methods come after and some steps of functionalization of nanoparticles are also presented because they are related with the procedure selected to be the best option for 5FU-MIP.

Finally, results are presented and discussed followed by conclusions, possible improvements and future perspectives.

2 State of Art

Cancer is a disease caused by both a genetic predisposition and environmental factors and there is no cure yet but some treatments are available [36]. Cancer occurs after normal cells have been transformed into neoplastic cells through alteration of their genetic material and the abnormal expression of certain genes. Neoplastic cells usually exhibit chromosomal abnormalities and the loss of their differentiated properties. These changes lead to uncontrolled cell division and many result in the invasion of previously unaffected organs, a process called metastasis [17].

Surgery and radiotherapy are the oldest treatment options for cancer, but since 1965 chemotherapy is available and now is widely used. Cytostatics are PhACs used in chemotherapy. They prevent the tumor growth and generally have a cytotoxic effect too leading cancer cells to death. A major problem is that these compounds aren't specific for cancer and important cells with rapid growth as marrowbone cells are affected causing adverse human health problems [17].

2.1 Cytostatics: 5-fluorouracil

According the *Anatomical Therapeutic Chemical* (ATC) classification system, cytostatic drugs are divided into 5 classes - alkylating agents, antimetabolites, plant alkaloids and other natural products, cytotoxic antibiotics and other antineoplastic agents. 5-fluorouracil (5-FU) is an antimetabolite discovered by Dr. Charles Heidelberger that hinders cell growth through inhibition of thymidilate synthetase, thereby obstructing thymidine synthesis and ultimately blocking normal DNA replication. It also causes the synthesis of faulty DNA and RNA through incorporation of 5-FU active metabolites in lieu of pyrimidine bases [3, 5, 19]. Chemotherapy dosing of 5-FU is currently based on body surface area, but Saif et al. point out this approach as limited use [37]. Various strategies have been developed to enhance the clinical activity of 5-FU, such as biochemical modulation, alterations in scheduling of administration, and the use of oral chemotherapy.

5-FU is 5-fluoro-2,4-(1H,3H)-pyrimidinedione (*Figure 3*), a fluorinated pyrimidine with the empirical formula $C_4H_3FN_2O_2$, a molecular mass of 130.08 g/mol and a *Chemical Abstracts Services* (CAS) number 51-21-8. For nearly 40 years, 5-FU was the only anticancer agent approved for use in the United States for the treatment of advanced colon rectal cancer (CRC) and adjuvant therapy of early-stage colon cancer [19]. Nowadays, 5-FU is also used for other solid tumors like breast, gastric, head and neck tumors and there are more patient-friendly prodrugs as for example tegafur and capecitabine (CAP). Both are orally

administrated and they have an efficacy similar to that of intravenous 5-FU, with potential advantages in terms of convenience and quality of life for the patient and cost-effectiveness as compared with intravenous chemotherapy. CAP is a fluoropyrimidine carbamate rapidly metabolized to the active substance 5-FU to a higher degree in hepatic and tumor tissues - where enzyme cytidine deaminase is expressed above normally. The simple conversion of CAP into 5-FU is exemplified in *Figure 3* [3, 5, 38, 39].

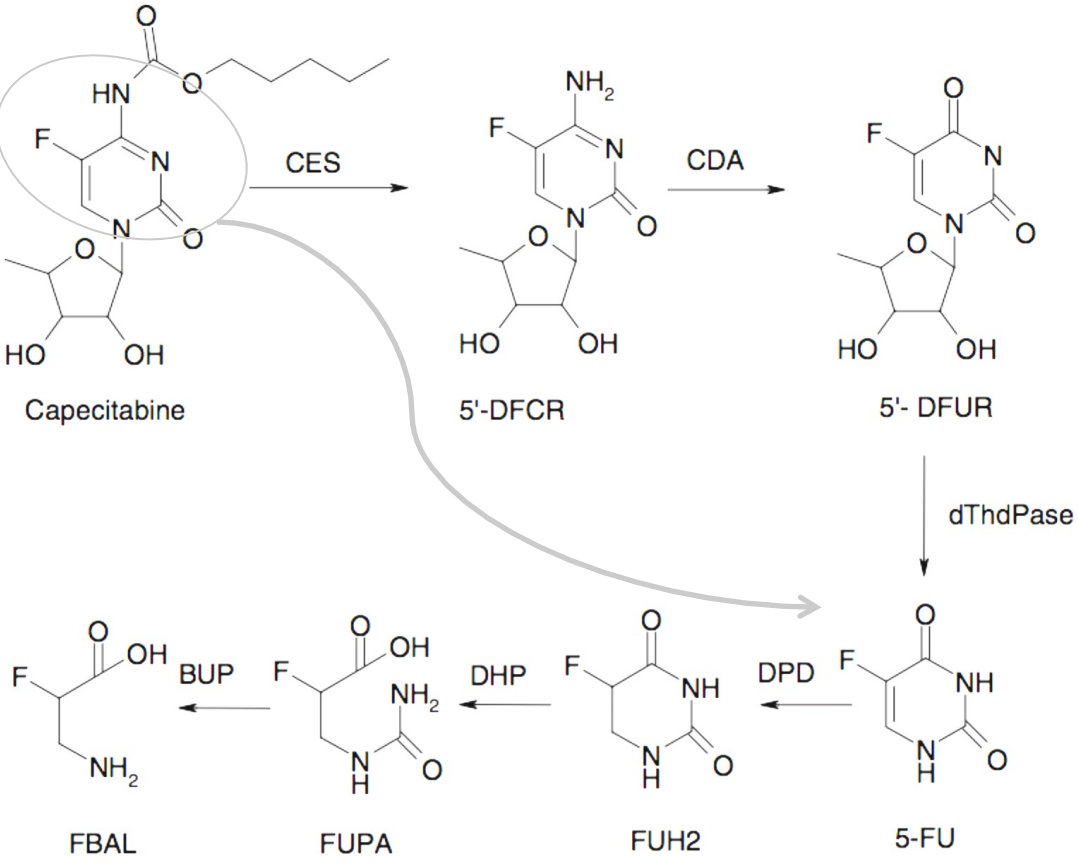


Figure 3. Bioactivation pathway of capecitabine. Enzymes: carboxylesterase (CES); cytidine deaminase (CDA); thymidine phosphorylase (dThdPhase); dihydropyrimidine dehydrogenase (DPD); dihydropyrimidinase (DHP); β-alanine synthase (BUP). Adapted from [38].

In 2009, the Swiss global health-care company (F. Hoffmann-La Roche) that sells 5-FU and CAP, published a report by J.O. Straub [5] about the environment risk of these two compounds. The report pointed out that 5-FU is persistent in surface water (half-life of more than 40 days) at 12 °C, but the final conclusions are that current use of CAP and 5-FU doesn't represent any evident risk to the environment. However, a more recent report from Kosjek et al. (2013) [3] that highlights the analysis, environmental occurrence, fate and transformation of 5-F have assumed, according to previous studies, that all the administered drugs aren't metabolized and the metabolism rates are 0.85 for 5-FU and 0.70 for CAP, being 15% of intact

5-FU excreted. They detected 5-FU at concentrations from 4.7 to 92 ng/L in samples of oncological and municipal wastewaters (WWs). Mahnik et al. detected the maximum concentration of 5-FU (124 µg/L) in hospital WWs [16]. These results and other publications found in literature mentioning the occurrence of 5-FU in WWs are included in *Table 1*.

Table 1. Ocurrence of 5-FU in wastewaters.

City/Country	Source	5-FU concentration	Reference
Vienna, **Austria**	Hospital WWs	20 – 122 µg/L	[21]
Vienna, **Austria**	Hospital WWs	8.6 - 124 µg/L	[16]
Switzerland	Hospital WWs	0.005 – 0.027 µg/L	[40]
Switzerland	Hospital WWs	7400 µg per day	[41]
Paris, **France**	Hospital WWs	0.09 - 4 µg/L	[42]
Ljubljana, **Slovenia**	Hospital and municipal WWs	0.0047 – 0.092 µg/L	[3]

Kosjek et al. listed in 2011 all studies reporting the occurrence of cytostatics in wastewaters (WWs) and in the environment [7]. However, Negreira and co-workers in 2013 concluded that the number of studies investigating and reporting the presence of cytostatics in water samples was still quite short and they alerted to the existence of degradation products, which in turn can be more toxic and persistent [1]. *Table 1* confirms a lack in information with just 6 reports indicating the occurrence of 5-FU in water systems, especially in hospital wastewaters which typically are not submitted to specialized treatment before discharge in water courses. Booker et al. also agree that there is little information about the presence of cytostatics in wastewaters and criticize that there is even less in respect to other matrices such as WWTPs effluents and surface waters. The two most monitored cytostatic drugs (cyclophosphamide and ifosfamide) have been found in surface waters, however, the authors highlight trough their prioritization method based on use patterns for hospitals that 5-FU and CAP are expected to be the most abundant cytostatics present in the aquatic environment but there aren't studies yet reporting their presence in surface waters. The prioritization method based on 2010 - 2012 consumption in NW England assuming just urinary excretion of the unchanged drug and the best removal rates in WWTPs predicted a river water concentration of 2.3 and 0.9 ng/L for CAP and 5-FU, respectively *(Table 2)*. CAP can also be fecal excreted and since CAP is a pro-drug of 5-FU, may contribute to 5-FU load [11]. Besse et

al. had conducted a similar prioritization study based on consumptions in France for years 2004 and 2008 and 5-FU with a predicted environmental concentration (PEC) of 0.8 ng/L wasn't on the prioritizing list of cytostatics but its pro-drug CAP was included, with a PEC of 2.9 ng/L [2]. However, the more recent prioritization study already mentioned, by Booker et al. (2014) based on data collected on England, considered both 5-FU and CAP plus 13 more cytostatics as priority contaminants [11].

Table 2. Consumption and predicted environmental concentration of 5-FU and CAP.

	Consumption (kg/year) (Local)	Excretion (%)	% after WWTP /biodegradation	PEC[1] (ng/L)	Reference
5-FU	1733.20 (France)	20	<91	<0.8	[2]
	22.99 (NW England)	18	85	0.9	[11]
CAP	5134.94 (France)	3	85	2.9	[2]
	357.00 (NW England)	3	85	2.3	[11]

[1]PEC - Predicted environmental concentration. PEC values are WWTP effluent values with a dilution factor of 10.

Besse et al. also presented a conservative PEC supposing a worst-case scenario of 100% excretion and 0% removal in WWTPs and the values obtained for 5-FU and CAP were 39.57 and 117.24 ng/L, respectively [2].

These concentration prediction studies are important but must be supported by studies of real analyses of cytostatics in the aquatic environment.

2.1.1 Analytical methods

The environmental detection of cytostatics, the production of new removal technologies or drug delivery systems for cytostatic compounds, its medical determination in human plasma and even degradation studies require analytical methods. The selection of the most suitable method will depend on the application, where the matrix and the necessary levels of quantification are deciding factors [17].

In environmental analysis, as concentrations are trace-level, it's required higher sensitivity, specificity and accuracy, which is possible using mass-spectrometry (MS) detection. The application of this technique to different cytostatic drugs analysis has been described providing mass spectral characterization of cytostatic compounds in some recent works [1, 22]. A complete review of analytical methods used for environmental monitoring of antineoplastic agents was published in 2003 by Turci et al. [23].

For pharmaceutical formulations, a valuable method for quality control, in agreement with pharmaceutical regulations, should be able to simultaneously determine the parent drug and its impurities and degradation products. Usually, separation techniques offering great selectivity, such as liquid chromatography (LC) or capillary electrophoresis (CE), are used coupled to MS because of its high selectivity and sensitivity that allows the detection of very low concentrations of impurities or degradation products [17].

Most of studies presenting analytical methods for 5-FU determination are related with medical applications as for example controlling the concentration levels in human plasma [38, 43-51]. It was made a compilation of studies reporting analytical methods for 5-FU quantification in water samples (*Appendix 2)*. As shown, most of them involve purification and/or concentration procedures, which allow reaching lower limits of detection. Kosjek et al. (2013) [3] obtained the lowest limit of quantification (0.54 ng/L) using a gas-chromatography (GC) coupled to MS with a prior solid-phase extraction (SPE). However, since the priority was to develop a high affinity 5FU-MIP and to test its removal capability at relatively high 5-FU concentrations, the search for a simple analytical methodology based on direct injection of the water samples is justified. Additionally, although advantages in terms of limit of detection levels reached are recognized, the extraction procedures prior to the injection are time-consuming. So, *Appendix 2* can be helpful in the future to select a method to be applied combined in 5FU-MIP adsorption studies at lower 5-FU concentration levels.

The equipment available during this thesis work was an high pressure liquid chromatography (HPLC) with a diode array detector (DAD) so, the most similar method available in the literature which uses this instrumental technique was reported by Mattos et al. (2013) [52]. This method was developed for the determination of 5-FU in polymeric nanoparticles and it is based in a methodology described in the United States Pharmacopoeia USP34 (2010) which uses ultrapure water as mobile phase. Mattos et al. used a reversed phase (RP) C18 column (Xterra Waters) with 5 µm particle size, 4.6 mm internal diameter and 250 mm length and they improved the resolution of chromatographic peaks adding acetonitrile (ACN) to the mobile phase. The chromatographic analyzes were performed in isocratic mode being the mobile phase composed of water and ACN (90/10, v/v) at a flow rate of 1.0 mL/min with a injection volume of 100 µL. The detection wavelength was 265 nm for 5-FU with a retention time of 3.5 min, when analyzes were processed at 25 ºC during. Mattos et al. obtained a maximum relative standard deviation (RSD) of 3.51% and a mean percent recovery of 99.95%, indicating respectively the precision and the accuracy of the method. The LOQ obtained was 32.78 µg/L. Although the polymeric nanoparticles of Mattos et al. aren't composed with same reagents of the 5FU-MIP, this method is the best approximation found in

literature and it has the advantage of being fast, simple and cost-effective with a low percentage of ACN used, which is also important to a reduced environmental impact.

2.2 Molecularly Imprinted Polymers (MIPs)

MIPs are a synthetic material usually prepared by copolymerization of a cross-linker and a functional monomer in the presence of a template, which is removed from the final product. In short, MIP is prepared by mixing the template molecule with functional monomers, cross-linking monomers and a radical initiator in a proper solvent. Then, the polymerization can be initiated with UV light or heat and the complexes formed between the template molecule and the functional monomers will be stabilized within the resulting rigid, highly cross-linked polymer. After polymerization, the template molecule is extracted and the result is a selective polymer capable of rebind the imprinted compound. To prepare MIPs with good recognition properties toward target compounds, proper selection of reagents is a crucial step [9, 26, 27, 29].

The selection of a functional monomer represents a critical step in the overall MIP production process. The complexity of multiple interactions among the pre-polymerization components makes the selection of functional monomers still a big challenge although recent computer simulation developments in chemistry can lead to a promising strategy to select optimal functional monomers capable of forming more stable complexes with templates [9, 26, 31]. Actually, the most commonly used monomer is the methacrylic acid (MAA) because it is capable to act both as hydrogen-bond donor and acceptor and it shows good suitability for ionic interactions. However, ß-cyclodextrins (ß-CDs) also have been pointed as good monomers due to their series of cyclic oligosaccharides with a hydrophilic exterior and a hydrophobic cavity forming complexes with the template through hydrogen bonds, Van der Waals forces, hydrophobic interaction or electrostatic affinity. Molar ratios between monomers and template shouldn't be too low resulting in few binding sites or too high producing non-specific binding capacity [9, 27, 29]. Nowadays, a more economic and environmental friendly methodology to study the best ratio is through an *in-silico* experiment.

The monomers functional group fixation around imprinted molecules is executed by the cross-linker producing a cross-linked rigid polymer. As functional monomer, the cross-linker should have a right dosage and commonly the selected ones are the ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM) and N,N-methylenebisacrylamide (MBAA) [9, 27, 28].

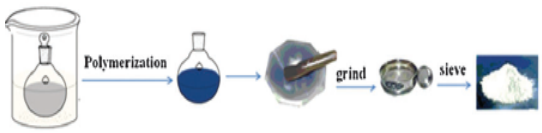
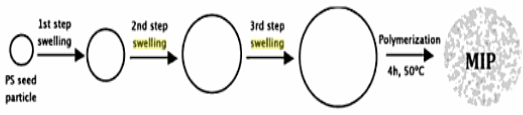
Finally, the ultimate critical reagent is the porogenic solvent and, in non-covalent polymerization processes (suitable for more templates), the most used are toluene, ACN and chloroform. However, the use of these porogenic solvents in MIPs preparation may constitute

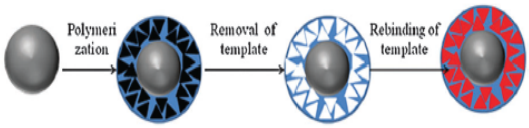
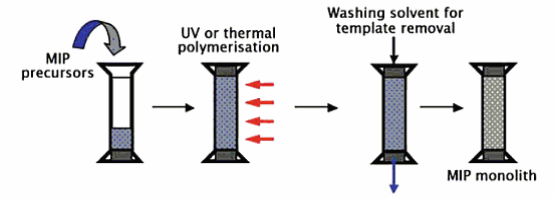
a constraint for their application in wastewater treatment because they tend to work poorly in aqueous media. Water can weaken the hydrogen interaction between templates and monomers. The optimal situation is an equality or approximation between imprinting conditions being a mixture of water and organic solvents as porogenic solvent an alternative. Also, hydrophilic monomers such β-CDs or supports with good hydrodynamic behavior where the MIPs can be grafted are also alternative solutions to the application in water samples [9, 10, 27, 28].

2.2.1 Methods for MIP synthesis

There are several ways of MIP production and the most known are presented in *Table 3*. This is a brief resume about the most common methods for MIP synthesis, whose details can be consulted in many specialized reviews [9, 10, 27, 31] and novel polymerization method associated technologies as controlled/living free radical polymerization (CLPR), block copolymer self-assembly, microwave-assistant heating and ionic liquid as porogen are documented by Chen et al. (2012) [9].

Table 3. Methods for MIP preparation [9, 31, 33].

Method	Advantages	Limitations	Scheme
Bulk polymerization	Simple and universal. Do not require particular skills or sophisticated instrumentation.	Tedious procedures of grinding, sieving, and column packing. Irregular particle (size and shape). Low performance.	
Suspension polymerization	Spherical particles. Highly reproducible results. Large-scale possibility.	Phase partitioning of complicates system. Water is incompatible with most imprinted procedures. Surfactant polymers are required.	
Multi-step swelling	Monodisperse beads of controlled diameter. Excellent particle for HPLC.	Complicated procedures and reaction conditions.	

Method	Advantages	Limitations	Scheme
Precipitation polymerization	Imprinted microspheres. Uniform size and high yields.	Large amount of template. High dilution factor.	
Surface polymerization	Monodisperse product. Thin imprinted layers.	Complicated system. Time-consuming.	
In-situ polymerization	One-step. In-situ preparation. Cost-efficient. Good porosity.	Extensive optimization is required for each new template system.	

The most popular method is bulk polymerization because it is fast and simple in preparation without requiring expensive instrumentation. The major disadvantage of this method is the time consuming operation, especially in the crushing and sieving processes, which also reduce the polymer yield. There are some different methods listed in *Table 3* that don't require the tedious steps after polymerization [9, 28, 29, 33].

Precipitation polymerization has been intensively reported because it is done with one single preparative step and allows a good control over particle size. However, MIPs produced by surface imprinting, have the imprinted templates situated at the surface of the material and can be applied in nanoparticles or in nanotubes showing good site accessibility and low mass transfer resistance, being considered the best suitable method to large templates as biological molecules, for example proteins [9].

Tan et al. prepared novel molecularly imprinted polymer nanoparticles (nanoMCN-MIPs) by covalent grafting of ofloxacin-imprinted polymer onto the surface of mesoporous carbon nanoparticles (MCNs) for selective removal of fluorquinolone (FQ) antibiotics in aqueous solution [8]. Ofloxacin (OFL) is a FQ antibiotic whose structure is not so different from 5-FU. This may suggest that the production of OFL-nanoMCN-MIP could be similar to the 5FU-nanoMCN-MIP, using 5-FU as template and doing some optimizations in the functional monomers and ratios according to 5FU-MIPs reports presented in the next section.

2.3 MIPs and 5-FU

There are at least four published reports about 5-fluorouracil-templated molecularly imprinted polymer (5FU-MIP) synthesis in the available scientific literature (*Table 4*). One is for 5-FU controlled delivery in biological systems [53], the other two for recognition and ultratrace analysis by medical sensors [54, 55] and the oldest one, from 2001, was developed for analytical purposes [56].

Table 4. Studies reporting 5-fluorouracil-templated MIPs (5FU-MIPs).

Reference	Method	Objective	Reagents	Results
[56]	Bulk polymerization	Analytical	1.4 mmol of 5-FU 1.4 mmol of BAP 2.8 mmol TFMAA 28 mmol of EGDMA 0.24 mmol of AIBN 19 mL of acetonitrile	Capacity factor (k') = 9.0 $I_f = 23$ [(a)]
[53]	Bulk polymerization	Controlled release device in biological fluids	2.0 mmol of 5-FU 16.0 mmol of MAA 20.0 mmol of EGDMA 103 mg AIBN	$I_f = 3$ [(a)]
[55]	Surface polymerization (grafted onto the surface of sol-gel immobilized)	Medical sensors	Equimolar dimethyl formamide (DMF) solution of: Melamine (mel) Chloranil (chl) 5-FU	$I_f = 6.4$ [(a)] 5-FU plasma concentrations with limit of detection of 48.4 ng/L
[54]	Surface polymerization (grafted onto different surfaces)	Medical sensors	5-FU:Ade-BTM:TTM 1:2:3 mol ratio	Limit of detection for 5-FU was 7280 ng/L

(a) Imprinting factor (I_f) = MIP response / NIP response.

Table 4 complements what was pointed out in previous section about the innovation in MIP production because the oldest studies used bulk polymerization as production method of MIP and the other two more recent applied surface polymerization being the 5FU-MIP grafted onto the surface of a support (sol-gel immobilized, indium-tin oxide or Au film-coated glass slides).

Kugimiya et al. verified that the MIP affinity for 5-FU was improved using 2-(trifluoromethyl)acrylic acid (TFMAA) as second functional monomer in comparison to the MIP produced using only 2,6-bis(acrylamido)pyridine (BAP). This may be attributed to the fact that the carboxyl group of TFMAA may have the ability to interact with the 1-position of the amide proton of 5-FU molecule. On the other hand, the affinity of 5FU-MIP for uracil

decreased when both the functional monomers were used in its production. Hence, it was concluded that TFMAA would interact with fluoride rather than the 1-position of the amide group of 5-FU [56].

Puoci et al. used a very similar monomer, which is the most common as it was already mentioned: the methacrylic acid (MAA) [53]. Accordingly to Kan et al., the MAA molecules are expected to interact with the carbonyl, amino and hydroxyl groups of 5-FU molecules through ionic or hydrogen bonds with their carboxyl groups, as shown in *Figure 4* [57].

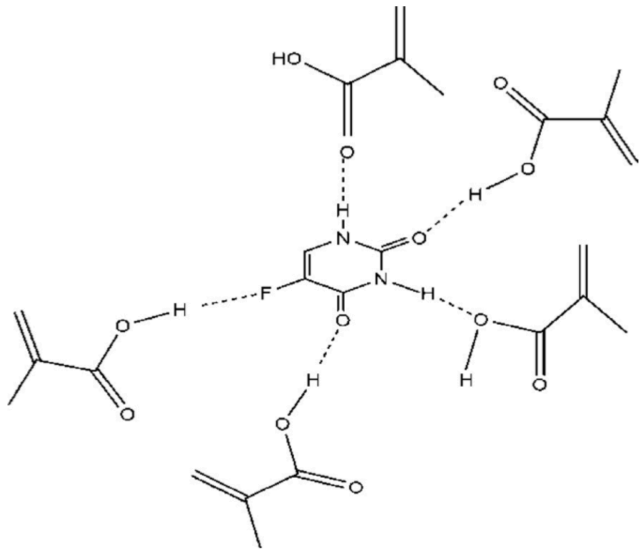


Figure 4. Illustration of interaction between 5-FU and MAA. Adapted from [57].

However, the imprinting factor (I_f) obtained by Puoci et al. was lower (3) than the one obtained by Kugimiya et al. (23), which indicates that TFMAA can be a preponderant factor to obtain good selectivity even for differentiation from similar compounds as uracil.

These previous studies create the basis of 5-FU drug delivery, analysis and therapeutic monitoring in biological systems. However, no such water treatment of 5-FU was obtained starting from MIPs. The conjugation of the OFL-nanoMCN-MIP technique of Tan et al. for water samples and the monomers here discussed, seems a good approach to achieve that purpose. One more time is relevant to repeat that there is already *in-silico* experiments developed for MIPs that could predict results and give better practical orientation. Developments in hardware and software resources and even in personal knowledge are required for that approach.

3 Technical Description and Discussion of Results

In this section, the proceeding for 5-fluorouracil molecularly imprinted polymer grafted onto mesoporous carbon nanoparticles (5FU-nanoMCN-MIPs) is briefly described and a detailed protocol can be consulted in *Appendix 3*.

3.1 Materials

Mesoporous silica nanopartciles (MSN) was synthesized with hexadecyltrimethylammonium bromide (CTAB), ammonium hydroxide 29% (aq. NH3) and tetraethylorthosilicate (TEOS) all purchased from Sigma-Aldrich and ethanol 99.9% (EtOH) and ultrapure water LC-MS grade from Merck. It was also used deionized water obtained in an ion exchange column.

3-aminopropyltriethoxysilane (APTES) from VWR, glucose and hydrofluoric acid 45 % (HF) obtained from VWR were used for mesoporous carbon nanoparticles (MCN) production. Toluene from Sigma-Aldrich was dried with phosphorous pentoxide obtained from Sigma-Aldrich. Then, for MCN functionalization, it was utilized nitric acid (HNO3), thionyl chloride (SOCl2), chloroform, anhydrous tetrahydrofuran (THF), allyl alcohol, 4-dimethyl aminopyridine (DMAP) and triethylamine (Et_3N) all purchased from VWR.

MIP production required 5-fluorouracil (5-FU), methacrylic acid (MAA); trifluormethylacrilic acid (TFMAA), trimethylpropanol trimethacrylate (TRIM) and azobisiobutyronitrile (AIBN) all with analytical grade from Sigma-Aldrich. Methanol, acetic acid and ethanol were obtained from Merck as acetonitrile (ACN) for HPLC analysis.

3.2 Equipment

Nanoparticles characterization was done by N_2 adsorption-desorption isotherms at 77 k on a Quantachrome Instruments NOVA 4200e. Fourier-Transform infrared (FTIR) analyzes were performed in a Bomem Arid-Zone MB-series and HPLC analyses in a Merck Hitachi Lachrom D-7000 system equipped with a high pressure pump, an autosampler injector, a diode array detector (DAD) and a Lichrospher RP C_{18} (250 mm x 4.6 mm, 5 µm) analytical column.

3.3 Experimental

3.3.1 MSNs synthesis

MSNs were produced based on the modification of Stöber synthesis, accordingly to [58]. In a polypropylene bottle, CTAB was dissolved in water forming micelles like in *Figure 5(A)*. Then, ethanol, aq. NH_3 and TEOS were added to a final composition of 0.41 CTAB : 11 aq. NH3 : 1 TEOS : 53 Ethanol : 344 H_2O. Ethanol dissolves TEOS (the silica source) and aq. NH_3 is the catalyst of silica condensation increasing the pH. The reaction was performed at 300 rpm for 16 h at 30 ºC. Possible interactions between silica and positive surfactant responsible for nanoparticles structure and porosity are in *Figure 5(B)*. S^+I^- is the most probably situation for this procedure.

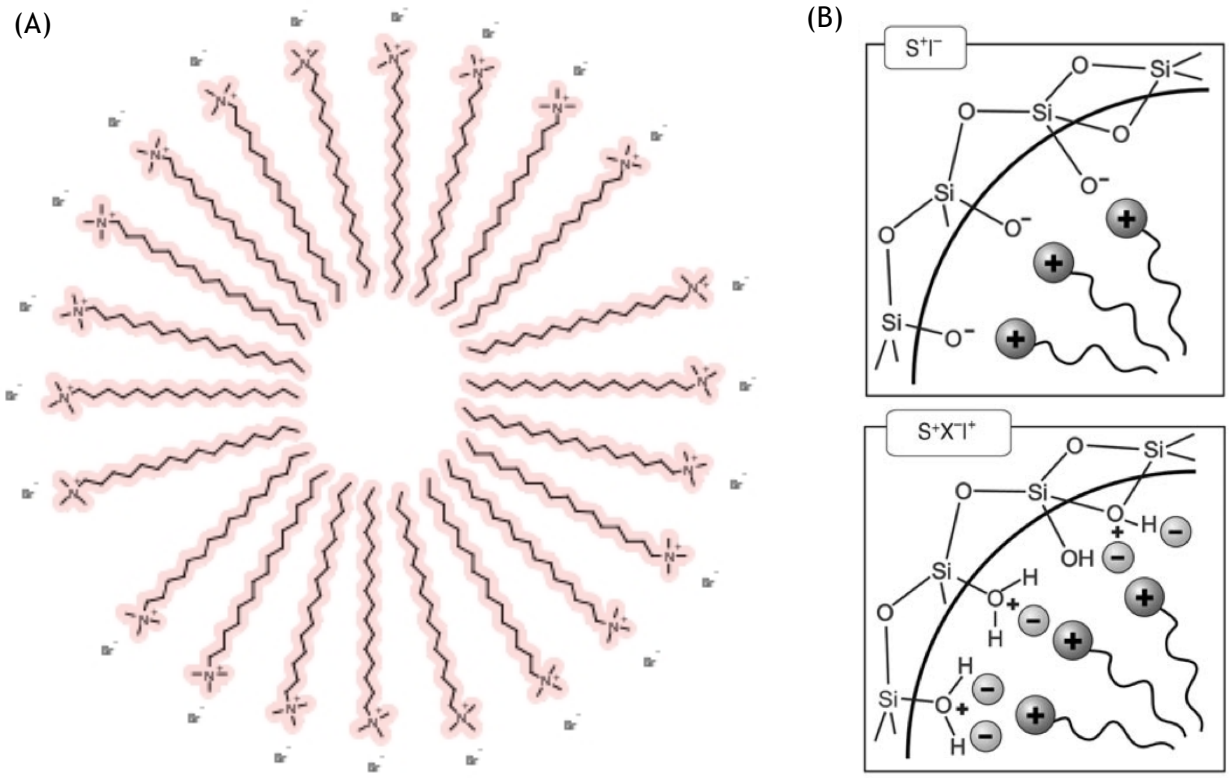


Figure 5. CTAB micelle designed on MarvinSketch Software v.6.2 (A) and two possible interactions between silica source and micelle adapted from [59] (B).

The precipitate obtained was filtered and washed with deionized water. Then, it was dried in an air oven at 85ºC overnight. The white powder was grounded finely and calcined at 550 ºC at a heating rate of approximately 3 ºC per minute to remove the surfactant. The type of MSNs obtained by this procedure is called MCM-48 first discovered by the scientists of Mobil Corporation and are characterized by a cubic structure usually detected by X-ray powder diffraction (XRD). However, the three-dimensional structure won't be discussed.

3.3.2 MCNs synthesis

Ryoo and co-workers [60] published the first study reporting the synthesis of MCNs from MCM-48 silica template by impregnation method with sucrose as carbon precursor and verified that the carbon product was not a perfect cubic replica of that template but a new cubic structure consisting of three-dimensional regular array of uniform mesopores 3 nm in diameter with a typical specific surface area of 1300 ~ 1800 m^2/g. The MCNs obtained by hard templating from MCM-48 MSN type were called CMK-1 [60-62] but in this work, there will not be XRD patterns to discuss the three-dimensional structure. Instead of impregnation method, there are being developed new techniques for the production of carbon materials through hydrothermal reaction of carbon precursors. This formation mechanism of carbon particles by hydrothermal carbonization is explained by Sevilla and Fuertes [63] in *Figure 6*. The hydrothermal reaction in the presence of a template controls the structure of the carbon particle.

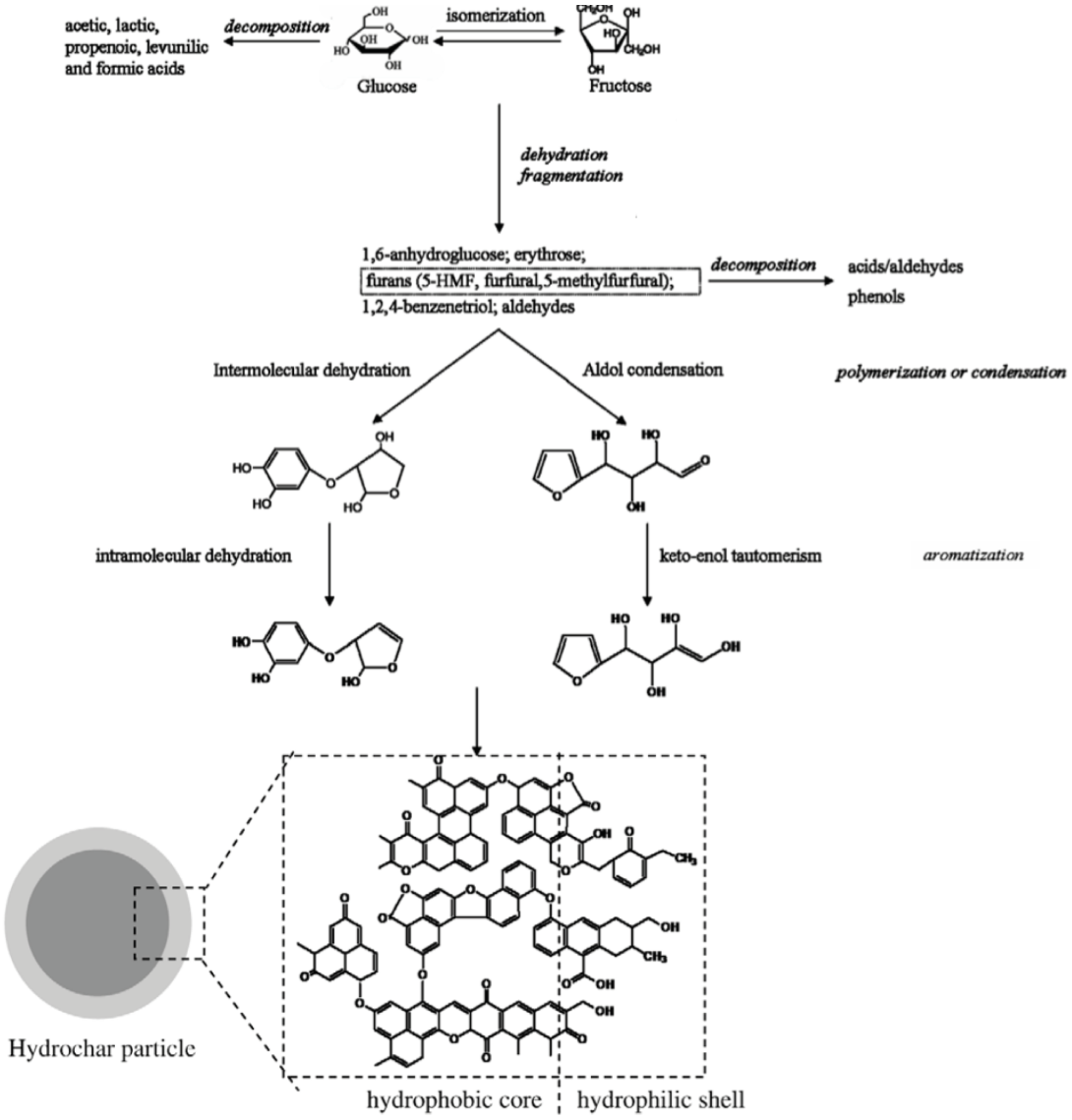


Figure 6. Mechanism of formation of carbon particles by hydrothermal carbonization [64].

The production of MCNs were based on Gu et al. that recently won the great challenge to prepare MCNs with uniform size below 200 nm, hydrophilicity for good water dispersibility and porous structure by conjugating hydrothermal synthesis and hard templating [65]: MSNs were dissolved in toluene and modified with APTES at 80 ºC for 12h under nitrogen atmosphere to produce amino-functionalized MSNs (NH_2-MSNs). The NH_2-MSNs particles were filtered and washed with abundant ethanol. After dried over night in a vacuum oven, they were dispersed in a glucose solution by ultrasonication and closed in a 160 mL teflon-lined autoclave (Parr Instruments). The hydrothermal reaction was performed at 180 ºC for 3 h. The brown powders obtained were filtered and washed with water and ethanol. Finally, the powders were carbonized at 900 ºC in inert atmosphere (N_2) for 1,5 h and treated with 10% HF to remove the silica template.

3.3.3 MCNs functionalization

MCNs were further functionalized with vinyl groups by several chemical reactions, based on the procedure of Tan et al. [8], represented in *Figure 7*.

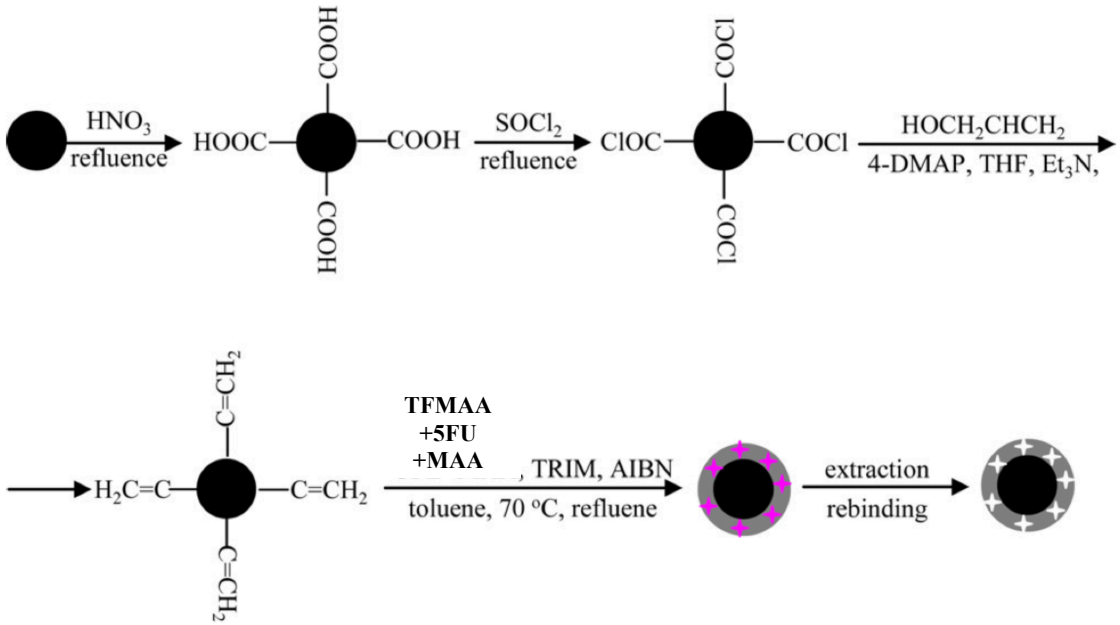


Figure 7. The routes of introducing vinyl groups and grafting MIP onto the surface of the MCNs. Adapted from [8].

First, MCNs were dispersed in a HNO3 solution (2 M) under ultrasonication for 30 min, followed by stirring at 65 ºC for 12 h. This process resulted in the production of carboxylic acids onto the surface of MCNs. The obtained MCNs-COOH were filtered and dried under vacuum. Then, MCNs-COOH were suspended in a mixture containing $SOCl_2$ and chloroform at

60 ºC for 24 h under reflux. The solid was removed and washed using anhydrous THF to remove the excess of $SOCl_2$ and then dried under vacuum to give MCNs-COCl. Then, MCNs-COCl in a solution of anhydrous THF were mixed with allyl alcohol, DMAP and Et_3N. The mixture was stirred at 50 ºC for 24 h and then filtered and washed using anhydrous THF. The solid was dried under vacuum, obtaining vinyl groups-functionalized MCNs (MCNs-CH=CH_2).

3.3.4 MIP synthesis

5FU-MCN-MIPs were prepared by the copolymerization of the monomers (MAA and TFMAA) and the cross-linker (TRIM) in the presence of 5-FU as a template and the MCNs-CH=CH_2. The particles were first suspended in anhydrous toluene under ultrasonication for 30 min and then 5-FU, MAA and TFMAA were added and stirred for 30 min to form TFMAA-5FU-MAA complexes by hydrogen-bonding interaction between them. TRIM and the initiator AIBN were afterwards added to the mixture that was further purged with pure N_2 for 10 min and left to react at 70 ºC for 24 h under stirring. The molar ratio was 1 5-FU : 1 TFMAA : 3 MAA : 4 TRIM.

The product was filtered and the resulting grey solid was removed and washed ultrasonically (10 min) with 200 mL MeOH/acetic acid (v/v = 9:1) solution. The obtained 5FU-MCN-MIPs were further rinsed with ethanol to remove the remaining acetic acid and then dried in vacuum. The MCNs grafted with non-imprinted polymer (MCN-NIPs) were prepared by the same procedures described above (including the same functionalized MCNs that waited 48h for MIP synthesis and material preparation) except with the particularity of not being added template in the polymerization process.

3.3.5 Analytical method

Chromatographic analysis was performed in the isocratic mode with a mobile phase of water/ACN (97/3, v/v), which was pumped at a flow rate of 1.0 mL/min. The sample injection volume was 100 µL and the method run time was 9 min. The temperature wasn't controlled.

Method performance was evaluated by estimating linearity, repeatability, accuracy and sensitivity. The linearity of the method was determined by calculating a regression line from the plot of peak area versus drug concentration for the 10 standard solutions in water (0.03, 0.1, 0.50, 2.00, 4.00, 7.00, 10.0, 15.0, 20.0 and 25.0 mg/L). The r^2 value was determined from the regression curve. Repeatability was estimated as instrumental (repeatability of measurement) and method repeatability (repeatability of sample preparation and analysis). The instrumental repeatability was determined as the RSD of six

consecutive injections of the same sample at three different concentration levels (0.5, 10 and 20 mg/L). Method repeatability results were also reported as RSD and for the same three concentration levels. The intraday variability (repeatability of sample preparation) was assess by testing three different standard solutions on the same day for each concentration level and the intermediate precision or interday variability was evaluated by analyzing the same standard sample on three different days. Sensitivity was expressed as LOD and LOQ. LOD was determined as 3-times the signal-noise ratio value for the lowest concentration and the LOQ 10-times the same value. Matrix effect was determined by comparing sample standards prepared in water that was in contact with MIP.

Finally, it was made a simple analysis adding 5-bromouracil (5-BU) to verify if this method was able to separate 5-BU and 5-FU. For this purpose, the run time was of 15 minutes.

3.3.6 Binding Experiments

In a thermostatic shaker, the binding capacities of the 5FU-MCN-MIPs and MCN-NIPs were evaluated at 20 ºC and 700 rpm without controlling pH. To obtain adsorption isotherms, in a 5 mL glass flask, 2 mg of particles were added into 3 mL of 5-FU standard solutions ranging from 4 to 200 mg/L. After 150 minutes, solutions were filtered (PTFE membrane, 25 mm, 0.22 µm) and analyzed by HPLC. To investigate the binding kinetics, it was selected a 5-FU standard solution of 25 mg/L and the same conditions of 2 mg of particles in 3 mL of the standard solution were applied for different times ranging from 5 to 150 min. After each corresponding time, solutions were filtered and analyzed by HPLC to calculate the adsorbed amount of 5-FU onto the MIP particles. In the same conditions, it was tested two different materials (inverted plastic test tube and a 24-well microplate) for adsorption studies, besides the glass flask, maintaining the ratio volume of solution/mass of particles.

3.4 Results and discussion

3.4.1 Textural properties

In this work, the first results obtained were related with the nanoparticles synthesized through N_2 adsorption-desorption isotherms. MSNs, NH_2-MSNs and MCNs were degasified and plunged in liquid N_2. Different pressures were applied and isotherms were obtained like the one in *Figure 8 to MSNs*.

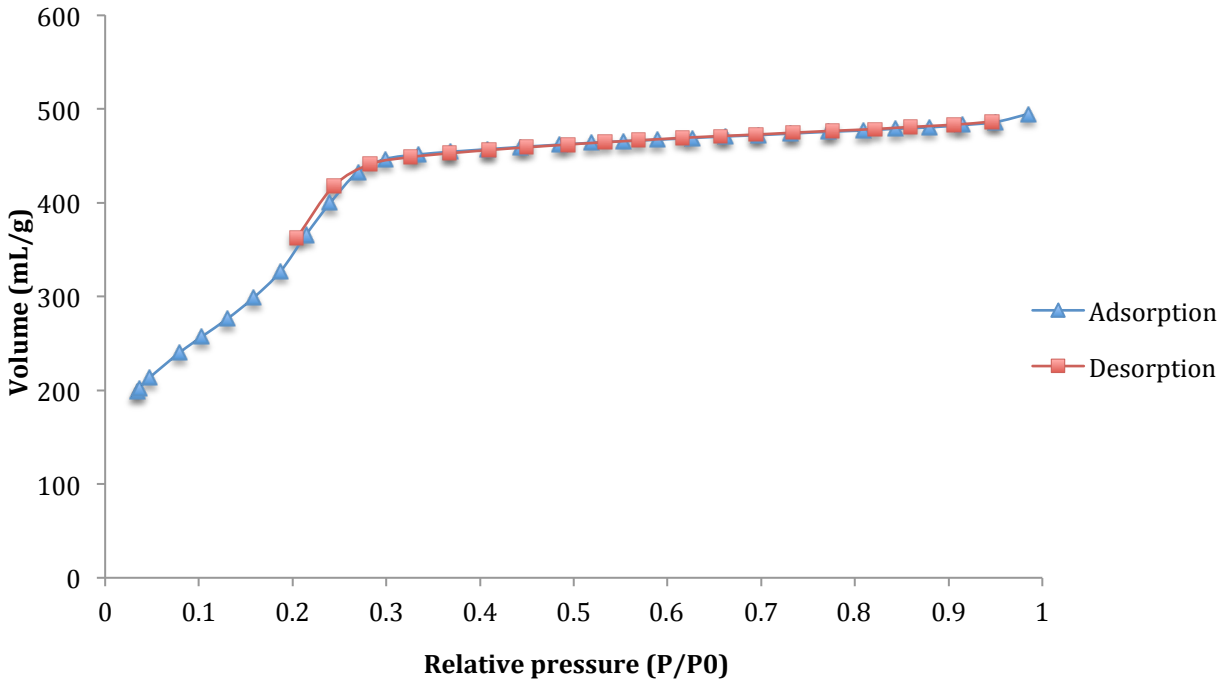


Figure 8. Nitrogen adsorption-desorption isotherm of MSNs.

Jomekian et al. [66] obtained an identical isotherm for their MCM-48 and they classified as a reversible type IV isotherm exhibiting a sharp pore filling step at p/p_0 0.2/0.3 which are characteristic of uniform pores accordingly to Jomekian et al. [66] and Ortiz et al. [67]. For the amino-functionalized MSNs and MCNs were obtained atypical isotherms, different of what was expected. However, to obtain the textural properties for the different materials it is necessary the application of some specific models. To determine the surface area, it was applied the Brunauer-Emmett-Teller (BET) [68] equation to the points of the adsorption isotherm at relative pressures (P/P_0) between 0.05-0.35 in the adsorption isotherm. The total pore volume was calculated by the total amount of N_2 adsorbed at the highest relative pressure [58].Finally, the pore diameter was determined by fitting Barrett-Joyner-Halenda (BJH) equation to the desorption isotherm points [69]. These methods applications gave some

properties of the three different materials analyzed. The surface area, pore volume and pore diameter results are presented in *Table 5*.

Table 5. Textural properties of particles.

	Surface area (m^2/g)	Pore volume (mL/g)	Pore diameter (nm)
MSNs	1504	0.81	3.162
NH_2-MSNs	383	0.24	3.121
MCNs	599	0.40	3.463

MSNs properties are similar to the ones obtained by Boote et al. [58] in the base study to this stage of synthesis, indicating that MSNs were successfully obtained with desirable properties of porosity and surface area. However, to NH_2-MSNs and MCNs the same didn't happened being the surface area and the pore volume too much lower than the ones achieved by Boote et al. [58]. It was expected to obtain all the values in the same order of magnitude of those obtained for MSNs with just a little reduce value for NH_2-MSNs due to the APTES functionalization at the surface of pores. The reduction of 1504 to 383 m^2/g of surface area and of 0.81 to 0.24 mL/g of total pore volume from MSNs to NH_2-MSNs may be due to an excessive ratio of APTES in NH_2 functionalization. As consequence, APTES can obstruct the pores of MSNs particles explaining why the pore diameter maintained almost the same value with a little reduction of 0.04 nm. This may indicate that the 0.24 mL/g NH_2-MSNs pore volume can be the volume of pores not obstructed and amino-functionalized. Beyond the ratio of APTES and MSN that must be studied to find the best fit, also a slower addition of APTES along the reaction should be compared with total addition in the beginning like it was made because there is the possibility of APTES molecules react with each other and form bigger complexes that obstruct the MSNs pores instead of react with silanol groups present in MSNs surface.

In respect to MCNs, the pore diameter is higher, which can explain some gain in pore volume and surface area compared to NH_2-MSNs but not all. Some of this surface area can be related to independent carbon particles formation totally separated from MSNs (*Figure 9*) as Gu and co-workers [65] verified when they proved the importance of amino functionalization to uniform the size and porosity of MCNs. It's probably that the final product obtained by this procedure is a mixture of MCNs and non-porous carbon microspheres that can be both functionalized but the good hydrodynamic behavior and the uniform size of the final product

won't be achieved, which may compromise the production of the correspondent MIP with nano and uniform size. Besides the optimizations mentioned to the amino functionalization, in this stage, the glucose quantity should be controlled in respect to the volume of pores of NH_2-MSNs determined by adsorption-desorption isotherm.

Nevertheless, the size of the different particles wasn't studied but transmission electron microscopy (TEM) or scanning electron microscopy (SEM) can be used to determine, as shown in *Figure 9,* the particle size and also complement the hypothesis discussed in the last paragraph. This involves economic resources and time, but it would be interesting results for future work.

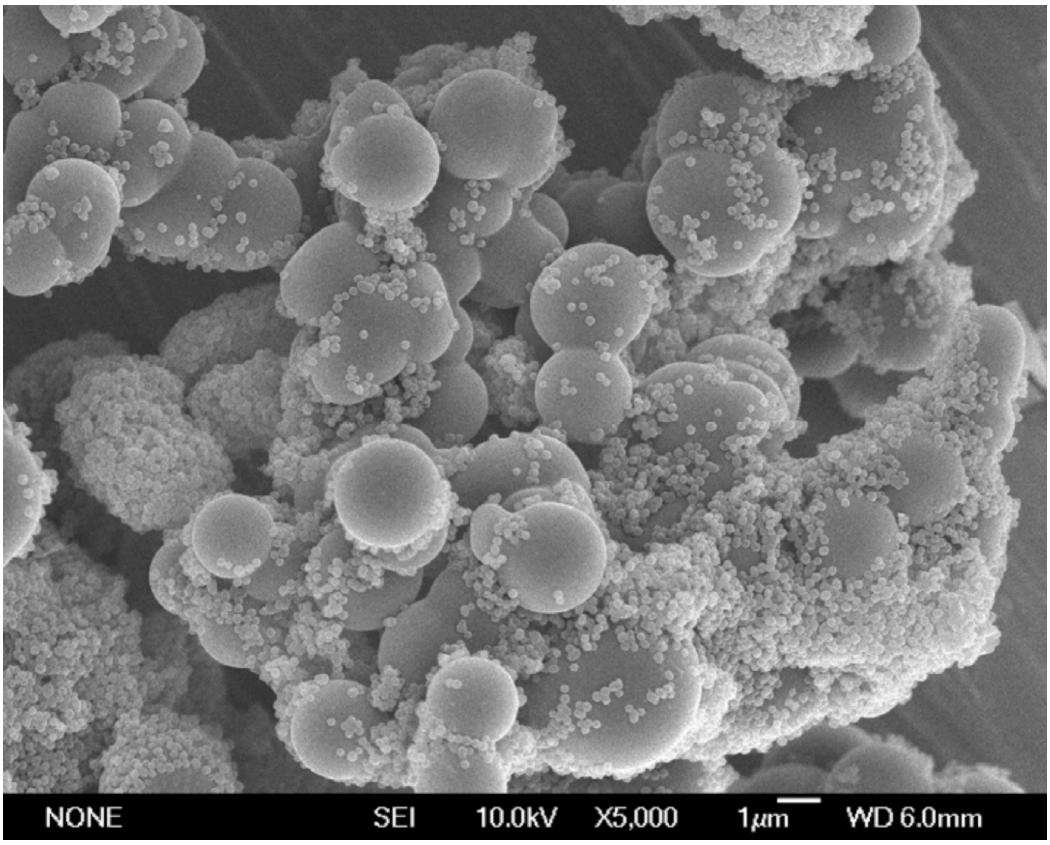


Figure 9. Enlarged SEM image of the mixture of MSNs and carbon microspheres via hydrothermal synthesis using blank MCM-48 as template (the small nanoparticles with size of 150nm are MSNs and the sizes of the totally separated carbon microspheres are about 2 μm). Adpated from [65].

3.4.2 Evaluation of Functionalization

The functionalization of MSNs and MCNs were followed by infrared spectroscopy comparing spectrums of the particles before and after functionalization. FTIR results are shown in *Figure 10.*

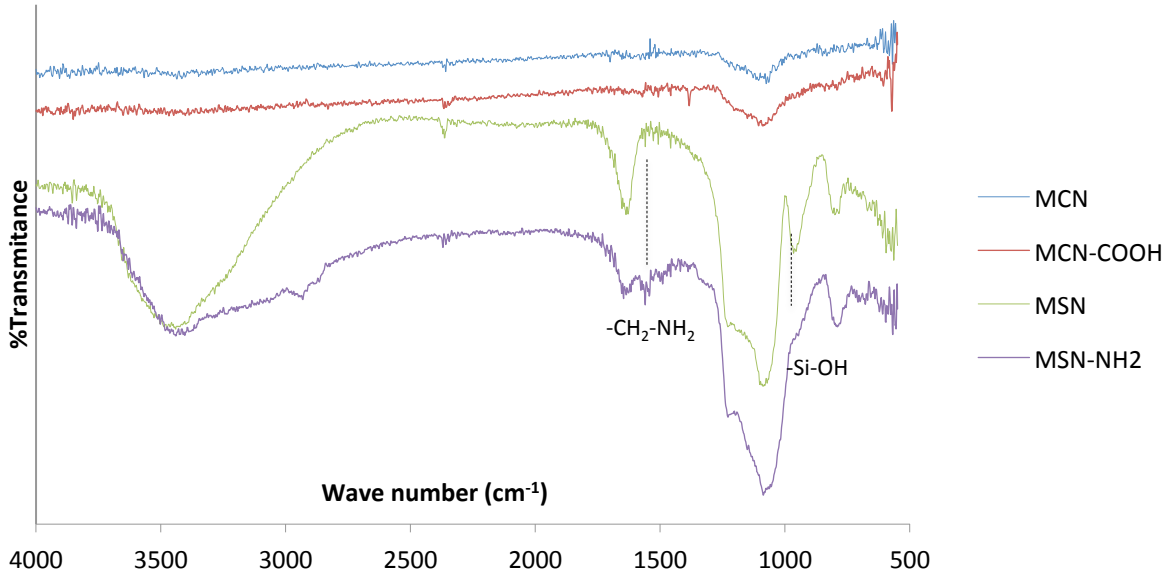


Figure 10. FTIR spectra of MSNs and its amino-funcionalzation and MCNs and its carboxyl functionalization.

Both MSNs and NH_2-MSNs exhibited a signal at 3500 cm^{-1} due to the O-H bond of the silanol groups and peaks at 1050 cm^{-1} and 750 cm^{-1} corresponding to the Si-O-Si stretching and bending vibrations, respectively. After amino grafting process, an apparent intensity decrease on the Si-OH vibration bands at 900-1000 cm^{-1} was observed while the new $-CH_2-NH_2$ signal at 1541 cm^{-1} emerged in NH_2-MSNs assigned to the NH_2 scissor vibrations. These are the two greater differences that prove the functionalization of MSNs with amino groups and they are concordant with literature [36, 65, 67].

The FTIR spectra of functionalized silicas showed bands at 2990 cm^{-1} due to the CH_2 stretching vibration modes of the hydrocarbon chain of APTES. The C-N stretching vibration is normally observed in the wavelength range of 1000-1200 cm^{-1}. However, this peak was not resolved due to overlaying with the Si-O-Si band present in the range of 1050-1150 cm^{-1}. Nevertheless, the peak of the NH_2-MSNs in this region is broader, indicating a possible overlap of peaks. In addition, the band of silinol groups (3500 cm^{-1}) is lower in NH_2-MSNs. The disappearance of this peak evidences the reaction of Si-OH groups and APTES. It should completely disappear after a perfect amine group attachment, but this didn't occur as was already confirmed by N_2 isotherms data. Thus, some covalent grafting of APTES on MSNs was clearly established by comparing FTIR spectra.

MCNs functionalization isn't easy to follow because the intensity of the predominant groups of carbon materials is too high and that's the reason why no difference is achieved comparing MCN and MCN-COOH spectra. The authors of the study, Tan et al. [8] in which this

work stage was based, also reported that it was not possible to control all the functionalization steps for the same reason. However, the functionalization was achieved because a final grey product was obtained. This grey color is related with the white polymeric material grafted onto the black carbon material. Here, a problem has occurred because this grey product was just obtained for the 5FU-MCN-MIP and not for the MCN-NIP (*Figure 11*). The last functionalization step of MCNs with vinyl groups rendered enough mass of particles to synthesize for two times the 5FU-MCN-MIP and the MCN-NIP. 5FU-MCN-MIP synthesis occurred as expected at first attempt so MCN-NIP production was done for three times but the black material was always obtained. Due to material limitations, each synthesis is performed with an interval of 2 days.

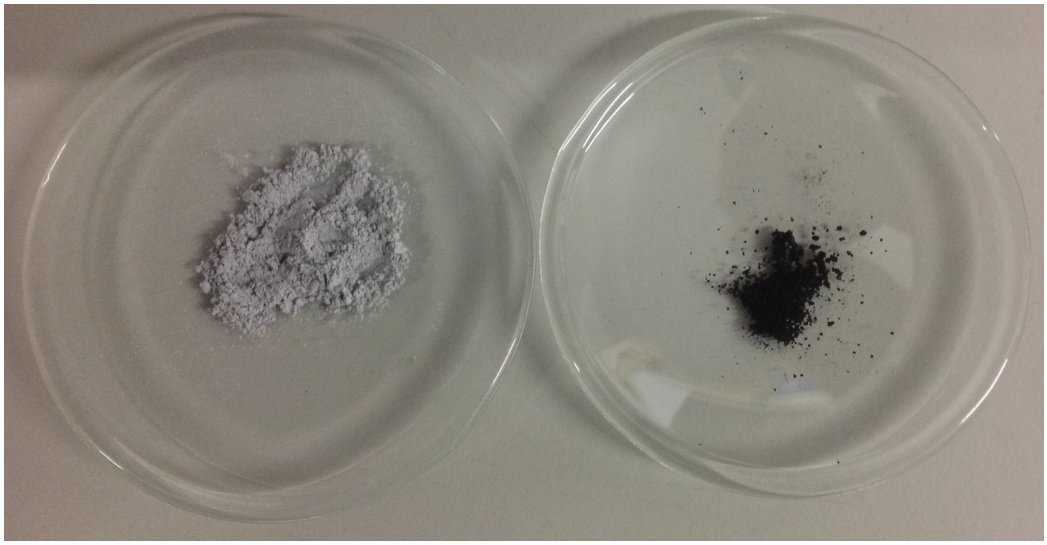


Figure 11. Colour difference of 5FU-MCN-MIP (left) and MCN-NIP (right) in aqueous solution.

The MCN-NIP maintained the black color of the carbon particles, but the final mass of the particles after polymerization (although almost 1 g inferior to the final mass obtained in 5FU-MCN-MIP synthesis) was superior to the 60 mg of MCNs added to the mix. This means that the final product isn't just the MCNs and for that reason it will still be called MCN-NIP. Probably, MCN-NIP are now particles with polymer inside its pores or particles surrounded by a thin layer of polymer that is not enough to change the final color of the product to grey. The expected NIP wasn't obtained maybe due to a loss of the vinyl groups of CH_2=CH-MCNs during the 48h of waiting (previous synthesis of 24h and material preparation) in a vacuum desiccator. Also, template can have a decisive role or MCNs functionalization presented low yield and most of the desirable final modified MCNs were used to 5FU-MCN-MIP synthesis.

This procedure is an innovate technique, which was developed for the first time at *LEPABE* during this thesis period and, for that reason, information about what to expect isn't available to compare MIP and NIP and conclude what is correct. So, to help understanding, simple adsorption tests were performed and the results are presented in the following sections.

3.4.3 Analytical method

Analyses were performed using ACN and water in many proportions, in isocratic mode and with the proportion of water/ACN raging from 97/3 (v/v) to 70/30 (v/v). The best separation and peak resolution was achieved when the proportion of water/ACN of 97/3 (v/v) was used. Under these conditions, the 5-FU peak was detected in approximately 4.5 minutes. *Figure 12* shows the chromatogram obtained at 265 nm (maximum wavelength of response to 5-FU, but 5-BU has a maximum response absorbance at 285 nm) when a mixture containing 25 mg/L of 5-FU and 5-BU was analyzed. A good separation between these two molecules was achieved. This allows for future work to test 5-BU as an analogue molecule in MIP production avoiding the occurrence of bleeding and also to perform cross reactivity tests to verify the real selectivity and specificity of the 5FU-MCN-MIP.

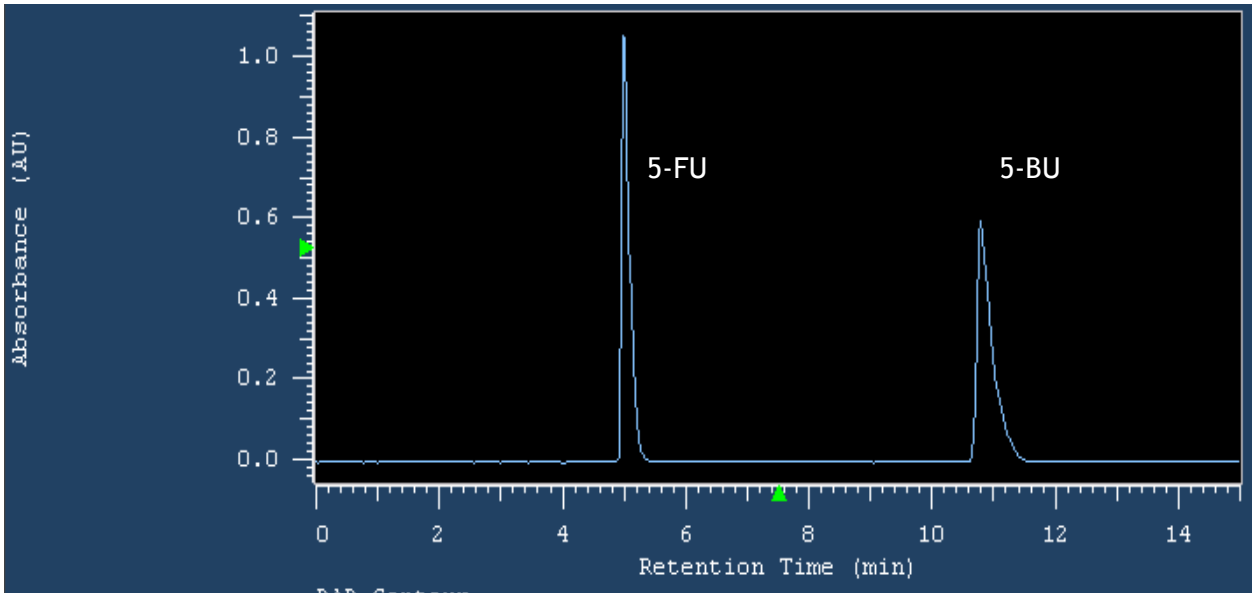


Figure 12. Chromatogram showing 5-FU and 5-BU (both at 25 mg/L) separation at 265 nm.

The validation parameters of the analytical method for determination of 5-FU in water samples are in *Table 6*. It was obtained a linear correlation with a LOQ of 0.03 mg/L. In respect to repeatability of measurement the maximum RSD achieved was of 1.74% for the

concentration of 10 mg/L (n=6). The maximum intra-day and inter-day variability was 0.73% and 6.58%, respectively. The maximum accuracy error was of 7%.

Table 6. Method development and validation parameters.

Parameters	
Mobile phase	97:3, v/v – Water:ACN
Flow-rate (injection volume)	1.0 mL/min. (100 µL)
UV	265 nm
Linearity (r^2)	0.03 – 25 mg/L (1.000)
LOD/LOQ (mg/L)	0.01/0.03
Repeatability of measurement (n=6) Conc. Range 0.5, 10 and 20 mg/L	0.37 - 1.74%
Repeatability of analytical method (n=3) Conc. Range 0.5, 10 and 20 mg/L	Intra-day: 0.21 - 0.73% Inter-day: 5.29 - 6.58%
Accuracy error Conc. Range 0.5, 10 and 20 mg/L	95 – 107%

3.4.4 Sorption kinetics

The sorption kinetic curves are illustrated in *Figure 13 and Figure 14*, for 5FU-MCN-MIP and MCN-NIP, respectively. The conditions of adsorption described previously were the same as Tan et al. [8] that, by the same procedure, synthesized an ofloxacin(OFL)-nanoMCN-polymer. They controlled the particles' size and can guaranteed their nano size. They obtained a maximum of 40 µg adsorbed at 120 minutes. The kinetic curve for 5FU-MCN-MIP has the same aspect to the one obtained by Tan et al. and the maximum adsorbed was 11 µg and occurred at 80 minutes. The kinetic curve behavior of MCN-NIP is irregular and duplicates should be performed to achieve conclusions.

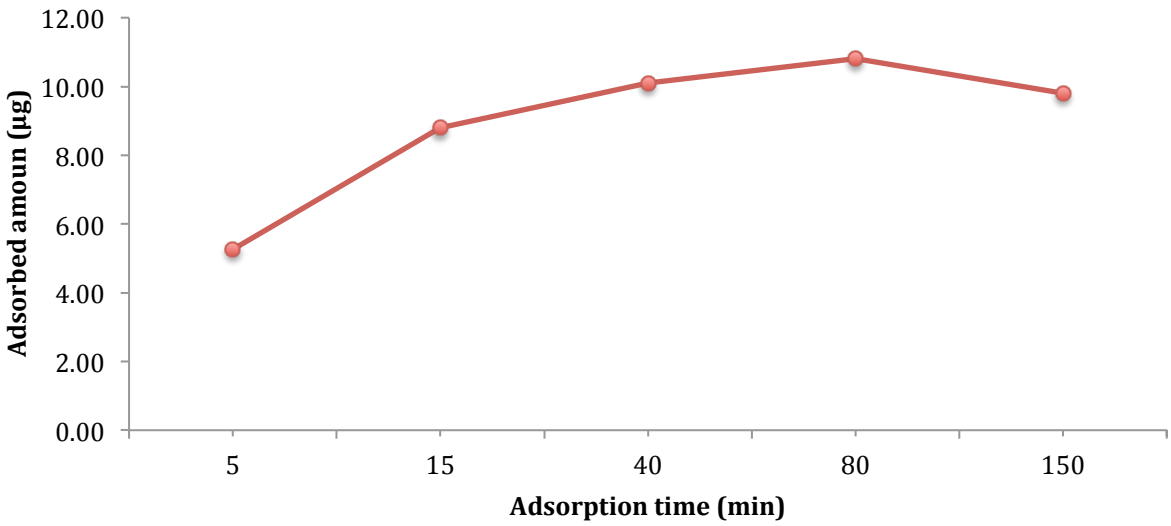


Figure 13. The adsorption kinetic curve for 5FU-MCN-MIP at 25 mg/L of 5-FU.

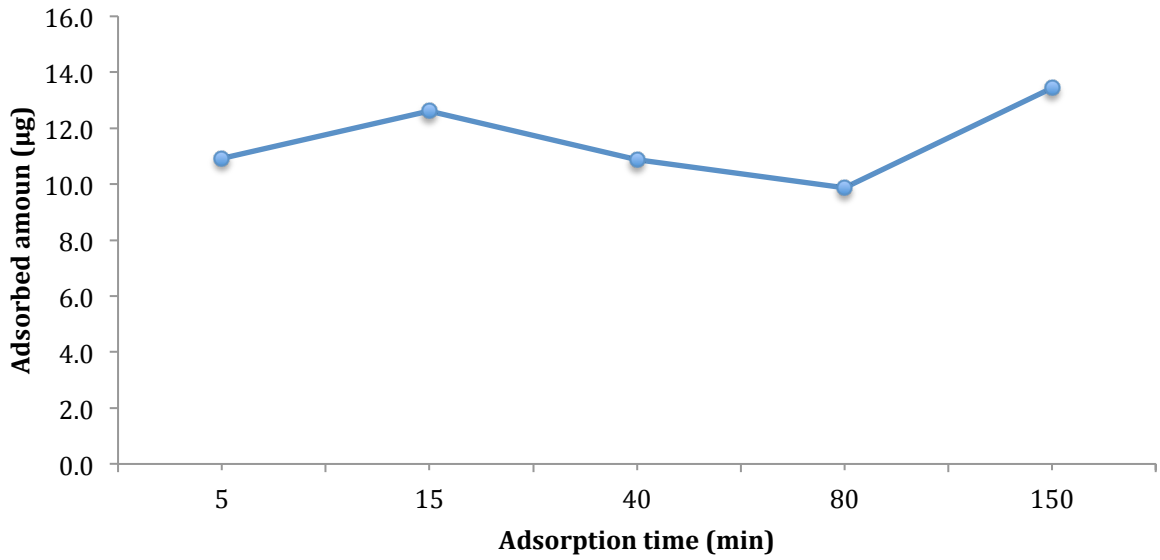


Figure 14. The adsorption kinetic curve for MCN-NIP at 25 mg/L of 5-FU.

3.4.5 Sorption isotherms

The adsorbed amounts for different concentrations are represented in *Figure 15 (A and C)* together with the % of adsorption for each concentration *Figure 15 (B and D)*. It can be concluded that maximum adsorption wasn't achieved to calculate the I_f and that MCN-NIP seems to present better adsorption capacity than 5FU-MCN-MIP.

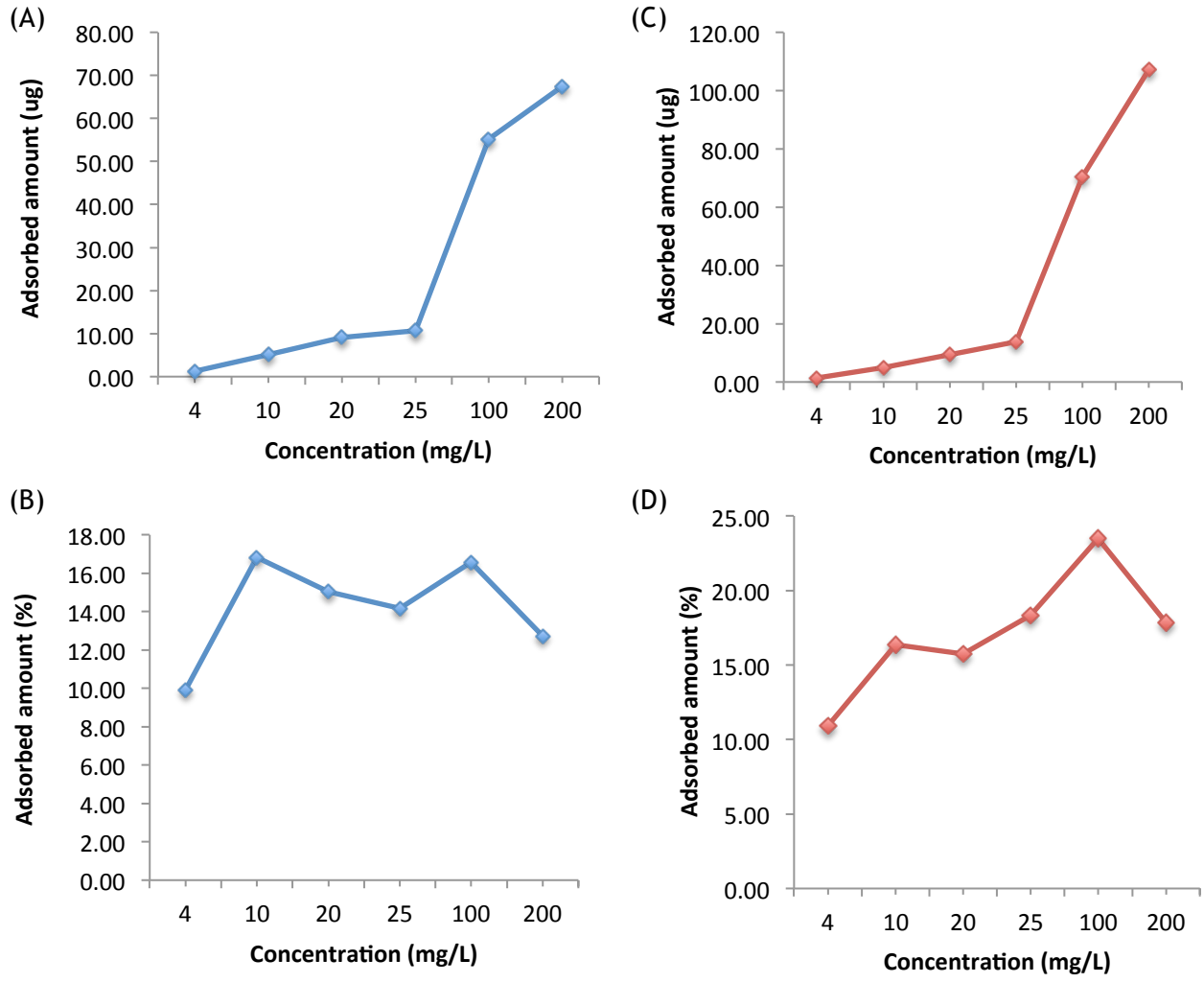


Figure 15. Binding results for the 5FU-MCN-MIPs (A and B) and MCN-NIPs (C and D) in aqueous solution.

The maximum adsorption capacities here obtained were 55 and 37 mg of 5-FU/g of polymer for MCN-NIP and 5FU-MCN-MIP, respectively. However, the real maximum adsorption capacity of the materials probably weren't obtained and new studies with larger concentrations range and duplicates should be performed. The great advantage of MIPs face to NIPs and even to carbon materials is verified in cross-reactivity experiments that should be performed when the aim of a study is to develop a MIP for selective removal of a compound from environmental waters [8] and this type of study should in future be performed to test if the 5FU-MCN-MIP here produced correspond to that advantage.

3.4.6 Adsorption conditions

Performing the adsorption experiments, it was verified that both products presented low density and as consequence, a great percentage of the 2 mg were afloat with practically no contact with solution even at elevated agitation velocity. Different adsorption conditions were tested and are illustrated in *Figure 16*.

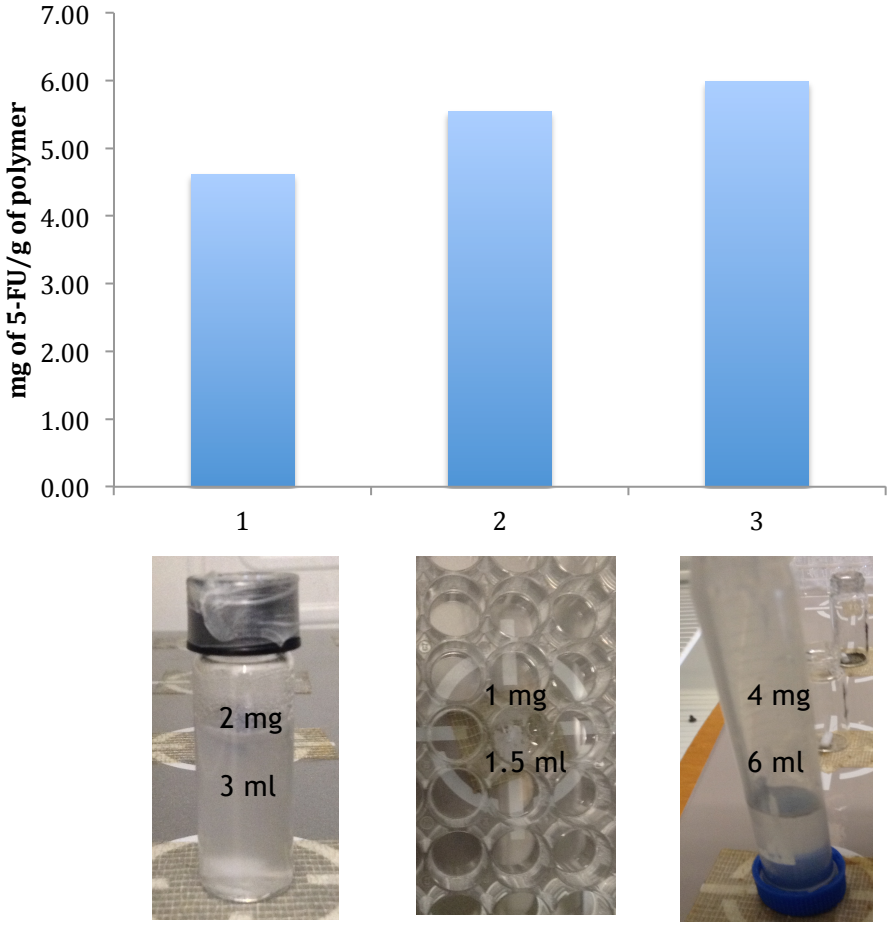


Figure 16. Adsorption comparison testing different adsorption conditions (1 - 5 mL glass falsk, 2 - 48-well plate and 3 - 15 mL test tube) tested for 5FU-MCN-MIP (25 mg/L of 5-FU).

The comparison between the three different conditions, supports that probably it is possible to improve the conditions of adsorption and the two new tested formula and material showed best results that the conditions tested in this work based in the same conditions used by Tan et al. for the OFL-nanoMCN-MIP. However, these tests do not give accurate conclusions because many variables were not controlled as agitation velocity and pH.

This could be one more optimization to join the amino functionalization and NIP production that should be done. Additional experiments should also be performed to verify the two great advantages of this type of material: selectivity and reusability due to easy regeneration.

4 Conclusions

Carbon nanoparticles synthesis by silica hard templating and hydrothermal reaction of a carbon precursor is a very innovative process in literature that was attempted in this work. First, new concepts were needed to understand about silica production by surfactant templating and functionalization with initial doubts if the amino functionalization was occurring just at external surface or inside the pores too. How to execute a hydrothermal reaction was also a challenge and different equipment resources were necessary from other laboratories, which required calendar availability. This was a time limitation and for future work, if all the resources of the procedure were available in *LEPABE,* this procedure can be totally executed in just two weeks, not including characterization tests.

The silica template, MSNs, was successfully produced with a surface area of 1504 m^2/g, a total volume of pores of 0.81 mL/g and a pore diameter of 3.2 nm. However, amino functionalization of silica particles weren't completely achieved as expected. FTIR and N_2 adsorption isotherms results suggest pore obstruction and just a few percentage of correct functionalization.

The procedure was continued to MCN synthesis and, at this stage, carbon microsphere separated from the silica template were probably produced. This occurred possibly because of a excess of carbon precursor face to a low pores volume of amino-functionalized MSNs. A possible explanation is that the particles obtained are a mixture of real MCNs with uniform size and porosity with non-porous carbon microspheres. A TEM or SEM picture could help to understand. The ratio of APTES/MSNs and the reaction conditions need further study. Also, the amount of glucose precursor used to obtain MCNs should be defined according to the total pore volume of NH_2-MSNs.

Besides all the limitations producing MCNs, these particles were functionalized and a 5FU-MCN-MIP was produced and simple tests of adsorption were conducted for first evidence of this technology's utility. The best adsorption capacity of 5FU-MCN-MIP was obtained at 200 mg/L of 5-FU after 150 minutes: 37 mg/g, but the maximum adsorption capacity seems not be achieved. The adsorption tests should be repeated to take better conclusions. The MCN-NIP production was done for three times and the black material was always obtained maybe due to a loss of the vinyl groups of CH_2=CH-MCNs.

It is important to emphasize that according to Tan et al. [8], the MIP developed herein may provide two important advantages over the carbon materials: selective sorption in aqueous solutions and repetitive usage due to easy regeneration. To test this properties, regeneration experiments and cross-reactivity experiments with similar interferences that can

be present in wastewaters should be performed [8]. The optimization of this technique can produce a viable technology to be applied in real wastewater treatment plants (WWTPs) like in *Figure 17* that exemplifies the final purpose of the work here started.

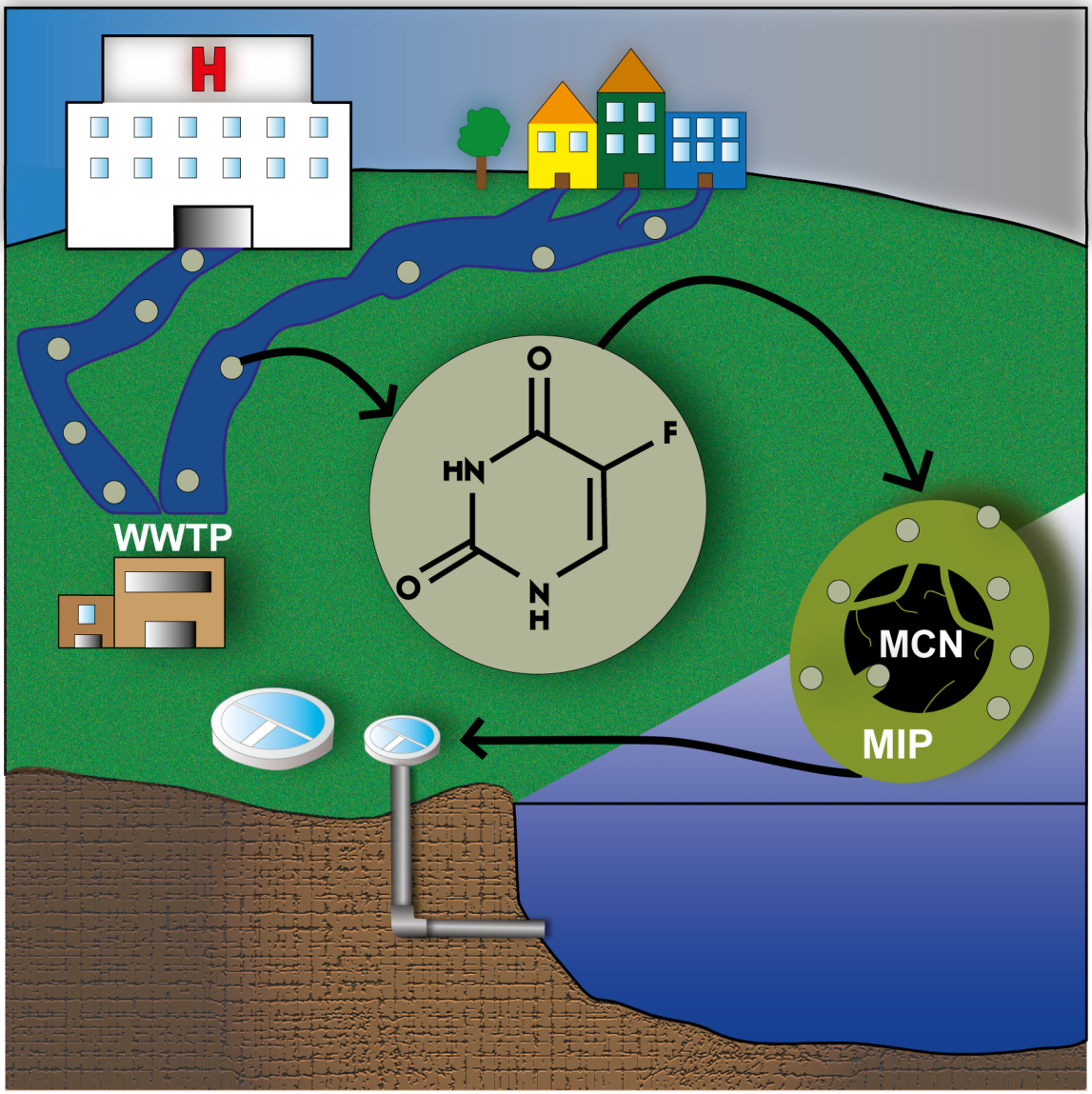


Figure 17. 5FU-MCN-MIP applied in wastewater treatment plant (WWTP).

4.1 Future work

In addition to all optimizations mentioned, an improvement that can be done is the magnetization of this technology as Hiratsuka et al. achieved for a bisphenolA-MIP. This will give advantages not only in the final application but also in the production process avoiding centrifugation and filtration procedures that are responsible for great yield losses.

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Appendix 1 - Metabolism pathway of 5-FU

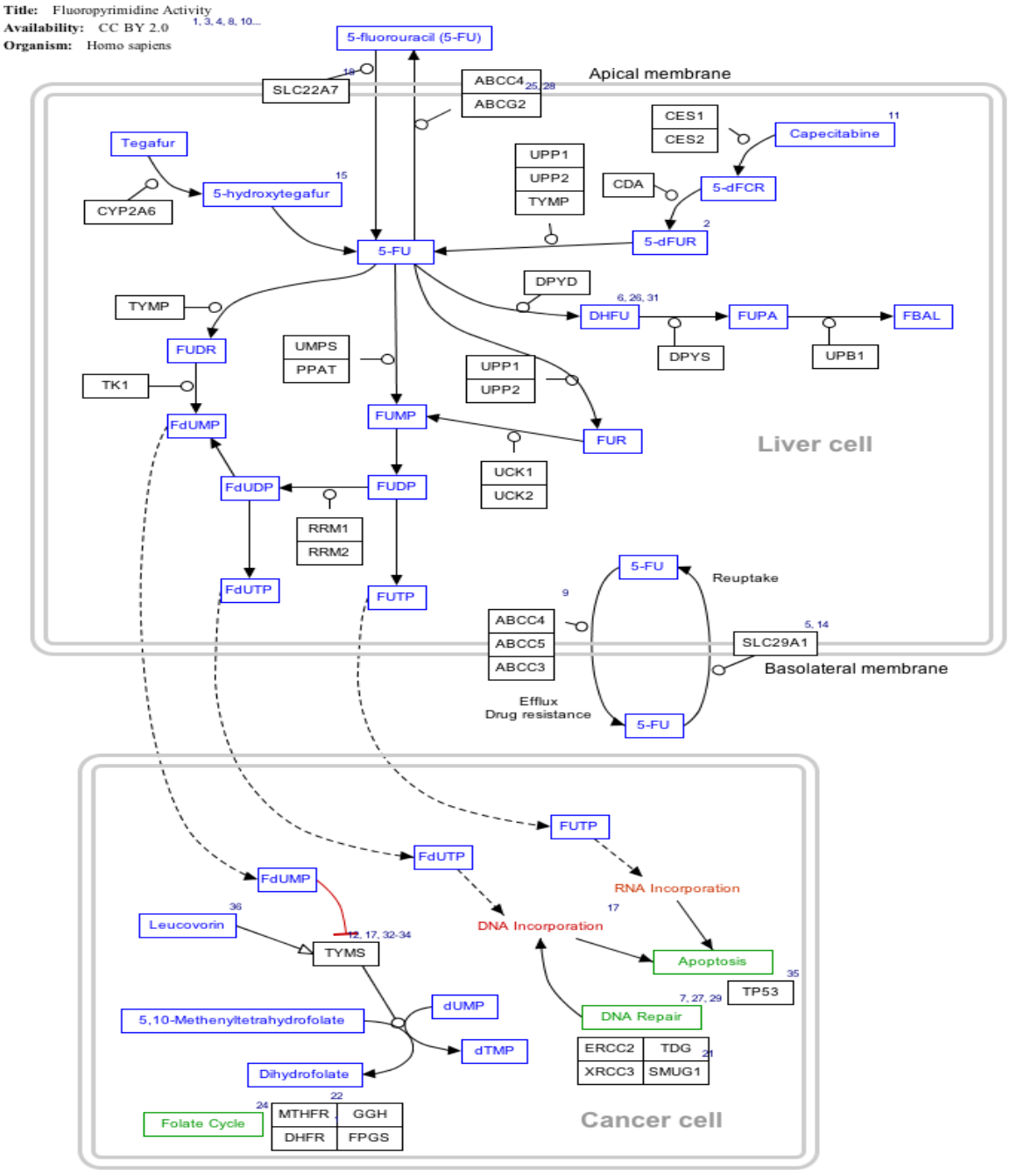

Image from Alexander Pico et al., available in:

http://www.wikipathways.org/index.php/Pathway:WP1601

Description: CAP is administered orally, readily absorbed from the gastrointestinal tract, and converted to the active 5-FU, which is further metabolized to inactive compounds as follows (the enzymes involved are given in parentheses): CAP (carboxylase) → 5'-deoxy-5-fluorocytidine (cytidine deaminase) → 5'-deoxy-5-fluorouracil (thymidine phosphorylase) → 5'-FU active compound.

Appendix 2 - Review of Analytical Methods

Analytical Method	Analyte	Sample type (Volume)	Extraction/Treatment	Separation	Detection	t_R (min)	Analytical parameters	Reference (Year)
SPE-HPLC-DAD	10 cytostatics including 5-FU	Wastewaters Surface waters (n/m)	**SPE** *Cartridge:* ENV+ (n/m); Conditioning: MeOH (3 mL) and KH_2PO_4 (3 mL, pH 5); Elution: H_2O/MeOH.	**HPLC** Column: Nucleosil C18 (5 µm, 250 x 4.0 mm); *Elution:* gradient; *Mobile phase*: (A) 0.01 mol/L KH_2PO_4 buffer (pH3), (B) MeOH. *Flow-rate: 1 mL/min;* Injection: 20 µL.	**UV** 267 nm	5.1	*LOQ: 50* µg/L *RSD: n/m* *REC: >90%*	*Kiffmeyer et al. (1998) [18]*
SPE-CE-UV	5-FU	Hospital wastewaters (50 mL)	**SPE** *Cartridge:* ENV+; *Conditioning:* MeOH (50 mL, pH 9), H2O (50 mL), 100 mM sodium acetate buffer (50 mL, pH 4.5); *Load:* 50 mL *Washing:* 50 mL H_2O *Reconstitution:* 30 mM disodium tetraborate buffer (100 µL).	**CE** *Capillary:* 56 cm x 75 µm (T=40°C); 30kV at 100 µA. *Buffer:* 80% 160 mM sodium borate buffer (pH 9.5) + 20% acetonitrile; *Injection:* 20 nL (30 mbar, 3.7s)	**UV** 265 nm	t_M = 21	*LOQ: 8.6* µg/L RSD: 0.7 – 9.5% Rec: 80 – 96%	*Mahnik et al. (2004) [16]*
SPE-GC-MS	5-FU and tamoxifen	Wastewaters (150 mL)	**SPE** *Cartridge: ENV+; Conditioning: MeOH 12 mL and 0.01 mol/L KH2PO4 (12 mL, pH 5); Load: 150 mL; Elution: MeOH (4 x 3 mL).* **Derivatization** *ACN (1 mL), 25% K2CO3 (100* µL) and 20% PFBBr (100 µL);	**GC** *Column:* capillary RTX-5 (60 m x 0.25 mm x 0.25 µm); *Flow rate:* 1 mL/min; *Injection:* 1 µL on-column (85ºC).	**MS** *Ionization mode:* EI and NCI.	42.9	**NCI** *LOQ:* 0.05 µg/L *RSD: 9%* *Rec: 69 - 77%* **EI** *LOQ:* 0.09 µg/L *RSD: 9%* *Rec: 69 - 77%*	*Tauxe-Wuersch et al. (2006) [70]*

			Incubation: 80ºC (1 h); *Reconstitution:* Toluene (1 mL evaporated to 200 µL); isooctane (1 mL). **Purification** *Cartridge:* SiOH*;* *Conditioning:* hexane:acetone (80:20 – v:v, 5 mL); *Washing:* toluene:hexane (15:85 – v:v, 8 mL); *Elution:* hexane:acetone (80:20 – v:v, 2 mL); *Reconstitution:* toluene (0.7 mL)					
SPE-HPLC-MS/MS	5 compounds including 5-FU	Hospital Wastewaters (50 mL)	SPE *Cartridge:* ENV+; *Conditioning:* MeOH (15 mL) and ammonium acetate buffer (6 mL, 10 mM); *Load:* 50 mL; *Washing:* H2O (2 mL); *Elution:* MeOH; *Reconstitution*: mobile phase (200 µL).	HPLC Cartridge: ZIC-HILIC (150 mm x 2.1 mm, 3.5 µm); *Mobile phase:* (A) 30nM ammonium acetate/ acetonitrile (2/3, v/v), (B) acetonitrile; *Flow-rate:* 0.2 mL/min; *Injection:* 2-6 µL	**UV** 267 nm **QqQ MS** *Ionization mode:* ESI(–) **HRMS** *Ionization mode:* ESI(–).	5.9	LOQ: 0.005 µg/L RSD: 1.7 – 5.6% Rec: 93 – 97%	*Kovalova et al. (2009) [40]*
SPE-HPLC-DAD	5-FU and 5-BU	Surface samples (2 mL)	**SPE** *Cartridge: N-VPDVB*; *Conditioning:* MeOH (1 mL) and water (1 mL); *Load:* 2 mL; *Elution:* MeOH/acetic acid (98:2, 2 mL) *Reconstitution*: mobile phase (1 mL).	**HPLC** *Cartridge:* C18 (250 x 4.6 mm, 5 µm) *Conditioning:* *Mobile phase* (95:5, v/v)*:* (A) 20 mM ammonium acetate buffer pH 4.7, (B) methanol; *flow rate*: 1.2 mL/min; *Injection:* 500 µL.	**DAD** 265 nm	4.5	LOQ: 25000 µg/L RSD: 2.1 – 14% Rec: 65 – 94%	*Resende et al. (2011) [13]*
SPE-GC-MS/MS	5-FU	WW SW (100 mL)	**SPE** *Cartridge:* ENV+ (1 g/6 mL for SW and 0.5 g/6 mL for WW); *Conditioning:* MeOH (6 mL) and H_2O (6 mL); *Load: 100 mL;*	GC *Capillary column:* 30 m x 0.25 mm x 0.25 µm; *Injection:* 1 µL on-column (250ºC).	**Ion trap MS** *Ionization mode:* EI	9.7	SW LOQ: 0.00054 µg/L RSD: 5.1 – 6.7% Rec: 91 – 95% WW LOQ: 0,0016 µg/L	*Kosjek et al. (2013) [3]*

			Elution: MeOH (3 x 2 mL); *Reconstitution:* ethyl acetate *(150* µL). **Derivatisation** MTBSTFA (30 µL).				RSD: 5.2 – 14% Rec: 53 – 81%	
LC-MS/MS	24 cytostatics and metabolites including 5-FU.	Standard solutions in water, methanol, DMSO or mixtures	*n/a*	**LC** Cartrigde: Purospher C18 (125 x 2 mm, 5 µm) Elution: gradient; *Mobile phase* (gradient elution): (A) Ultrapure water, (B), methanol, (C) modifier: 0.1% of formic acid; *Flow rate:* 0.2 mL/min; *Injection:* 10 µL	**QqQ MS** *Ionization mode:* ESI(-)	3.25	*LOQ: 16.6* µg/L RSD: 5.0 – 5.3% Rec: n/m	*Negreira et al. (2013) [1]*

n/m: not mentioned; n/a: not applied; LC: liquid chromatography; MS: mass spectrometry; QqQ: triple quadrupole; SPE: solid phase extraction; LOQ: limit of quantification; RSD: relative standard deviation; Rec: recovery; HPLC: High performance liquid chromatography; N-VPDVB: N-vinylpyrrolidone-divinylbezene; MeOH: methanol; WW: wastewaters; SF: surface waters; t_R: retention time; t_M: migration time; HILIC: hydrophilic interaction liquid chromatography; EI: electron impact or electron ionization; NCI: negative chemical ionization; ESI: electrospray ionization; DAD: diode array detection; HRMS: High resolution mass spectrometry; GC: gas chromatography;

Appendix 3 - Table Protocol

Step	Time	Procedure	Objective/Stage
1	1 h	Dissolve 1.2 g of CTAB in 50 mL of ultrapure water in a polypropylene bottle.	**Mesoporous silica nanoparticles (MSNs) preparation**
		After all surfactant is dissolved, add 25 mL of ethanol (stir 5 min), 10 mL of aq. NH_3 (29%) (stir 10 min) and finally add 1.8 mL of TEOS.	
	16 h	Stir at 300 rpm for 16h.	
2	30 min.	Filter off the precipitate and wash with deionized water.	
	12 h	Dry the white powder in air oven at 85 ºC overnight.	
3	30 min.	Transfer the dried powder from the filter to a ceramic capsule (pre weighted) and ground finely.	
	16 h	Calcine it in static air at 550 ºC (heating rate of 3 ºC/min). Let it cool down.	46h 2 days
4	20 min.	Add the MSNs obtained (dried) in 100 mL of dry toluene containing 1.2 mL (1.14 g, 5.13 mmol) of APTES.	**MSNs to MCNs**
	12 h	Under dry N_2 atmosphere, heat at 80 ºC for 12 h.	
5	30 min.	Filter the NH_2-MSNs and wash with abundant ethanol.	
	12 h	Dry at 100 ºC in vacuum oven over night.	
6	6 h	Disperse **0.5 g** of NH_2-MSNs in **40 mL** of a glucose solution (1.25 M) in a Teflon-sealed autoclave at 180 ºC for 3 h.	
7	1 h	Remove the powders by filtration and wash with water and ethanol.	
	14 h	Carbonize at 900 ºC in N_2 for 1.5 h.	
8	15 h	Treat with 10% HF at room temperature.	64h 4 days
9	3 h	Wash with distilled water and dry at 120 ºC.	

Step	Time	Procedure	Objective/Stage
10	30 min.	Then, add 1 g of MCNs into 250 mL of HNO_3 (2 M) under ultrasonication for 30 min.	**MCNs-COOH**
	12 h	Stir at 65 ºC for 12 h.	
11	5 min.	Remove the MCNs-COOH by filtration.	
	3 h	Dry under vacuum.	16h 2 days
12	10 min.	Suspend 0.4 g of MCNs-COOH in a solution of 10 mL of $SOCl_2$ and 30 mL of chloroform.	**MCNs-COCl**
	24 h	Heat at 60 ºC under 24 h under reflux and N2 atm.	
13	20 min.	Remove the solid and wash with anhydrous THF.	28h 2 days
	3 h	Dry under vacuum.	
14	15 min	Use 0.2 g of MCN-COCl in 30 mL of anhydrous THF to mix with 1.16 g (1.4 mL) of allyl alcohol, 0.244 g of (4-dimethylamino) pyridine and 6.06 g (8.4 mL) of triethylamine.	**MCNs-CH=CH$_2$**
	24 h	Stir at 50 ºC for 24 h.	
15	15 min.	Remove by filtration and wash with anhydrous THF.	
	3 h	Dry under vacuum	28h 2 days
16	30 min.	Suspend 60 mg of the MCNs-CH=CH_2 in 50 mL of anhydrous toluene in a 100 mL round-bottom flask under ultrasonication for 30 min.	**5FU-nanoMCN -MIPs**
	30 min.	Add 1 mmol (0.13 g) of 5-FU, 3 mmol (0.26 g) of MAA and 1 mmol (0.14 g) of TFMAA. Stir for 30 min.	
	5 min	Add 4 mmol (1.4 g) of TRIM and 30 mg of AIBN.	
	24 h	Purge the mixture with N_2 for 10 min and wait 24 h under 70 ºC under stirring.	
17	2 h	After filtration, wash the solid ultrasonically with MeOH/acetic acid (9:1).	30h 2 days
18	3 h	Clean with ethanol and dry under vacuum.	